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Colophon

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Introduction

Chapter 1. Introduction

Antibiotics provided humanity resilience to the majority of bacterial infections. It thereby altered the natural course of most infectious diseases and saved millions of lives. One could argue that antibiotics are the most significant development in modern medicine. An important trade-off is however, the emergence of antimicrobial resistance, and a diminished and perturbed microbiota, resulting in an increased susceptibility for *Clostridioides difficile* infections and Western (lifestyle associated) diseases [1-3]. The World Health Organization (WHO) has declared antimicrobial resistance one of the greatest challenge to global public health today, compromising the treatment of common bacterial infections [4]. More specifically, the Centers for Disease Control and Prevention (CDC) has outlined and prioritized the threats posed by specific multidrug Resistant Organisms (MDRO) of which drug resistant *Neisseria gonorrhoeae*, *Candida auris*, carbapenem resistant Enterobacterales and *C. difficile* were the most urgent [5].

To understand the role of the microbiota in defence against various infectious and non-infectious diseases, we need to define and measure the human microbiota by well accepted and standardized techniques, including methods to determine the function of the microbiota. This thesis focusses on the significance and possible interventions of the gut microbiota of patients colonized with antibiotic resistant bacteria or patients suffering from multiple recurrent *Clostridioides difficile* infection. This introduction summarizes the basic knowledge on the human microbiota of healthy and diseased individuals, the worldwide problem of increased antibiotic resistance and the threat of antibiotic-associated *C. difficile* infections.

The microbiota

The human gut microbiota

The human body houses a diverse microbial ecosystem, consisting of bacteria, archaea, fungi, viruses and parasites, together called the 'microbiota'. Because bacteria reach the highest density, the bacterial fraction of the microbiota is most studied. It is estimated that the ratio of bacterial to human cells in an adult body is 1:1 [6], and the collective bacterial genome contains 450-fold more genes than the human genome [7]. Each body site (e.g. gut, skin, vagina, stomach, oral cavity) has a different microbiota composition. With 10^{10}-10^{11} bacterial cells per gram feces, from approximately 1000 different species, the colon contains the largest number of bacteria [8]. The bacterial part of the gut microbiota is largely composed of two groups at the phylum level, the obligate anaerobic Bacteroidetes and Firmicutes [9]. In addition, the gut microbiota comprises of members of the Actinobacteria, Verrucomicrobia, Fusobacteria and Proteobacteria phyla [9]. It is important to realize that classification to phylum level gives a rather simplistic view of an extremely complex ecosystem *(Figure 1)*. Humans belong at phylum level to the Chordates, just like a Komodo dragon and a dolphin for example. Subsequently, the microbiota can be further subdivided at different levels; Phylum, Class, Order, Family, Genus and (sub) species *(Figure 1)*.

In general, it is believed that colonization, competition and engraftment of the human microbiota starts during labour. However, the "sterile womb" paradigm is currently challenged by several studies reporting bacterial communities in the intrauterine environment by next generation sequencing [10]. Others have found that viable bacterial colonization is highly limited in the foetal intestine or placenta, however can contain potential pathogens [11,12]. Still, the consensus is that current scientific evidence does not support the existence of a foetal microbiota [13], and that the finding of bacteria in the intrauterine environment is probably explained by contamination (e.g. of the kit, background DNA) [14,15]. Irrespective of the precise starting point of colonisation, birth gives microbiota development a boost. The human microbiota is acquired both vertically (via vaginal birth from mother) and horizontally (from the environment). Consequently, birth mode (vaginal or caesarean section) has significant effects on the microbiota development and composition [16]. In addition, early life microbiota succession is

influenced by, gestational age and breastfeeding. At two to three years of age, microbiota composition becomes more stable and adult-like. [17,18] The adult-like gut microbiota is functionally more complex and is structured to metabolize plant-derived polysaccharides from the diet [19]. The primary determinant of the microbiota composition is body site or habitat [9,20,21]. Within habitats, the interpersonal variability is high, whereas individuals exhibit only minor temporal variability [21]. Although the microbiota is constantly exposed to environmental stressors, its composition and function in an individual are relatively stable against most perturbations [22]. This resistance to change is described as resilience; the property of a microbial community that defines how fast, and to what extent, it will recover its initial functional or taxonomical composition following a catastrophic perturbation [22]. Still, minor changes in environmental factors such as diet, medicine use, season, travel or house-hold contact can affect the microbiota [23-25]. A recent study showed for instance that a large percentage of non-antibiotic drugs can inhibit the growth of certain bacteria, or even complete bacterial classes [24]. In fact, 24% of human drugs, amongst all therapeutic classes, inhibited bacterial growth of at least one bacterial strain [24]. Surprisingly, the chemically diverse antipsychotics were overrepresented as microbiota effectors. One could even speculate that regular use of pharmaceuticals nowadays may contribute to the decrease in microbiota diversity of the modernized human populations [26,27].

Kingdom	Bacteria						Animalia	
Phylum	Firmicutes	Bacteroidetes	Actinobacteria	Verrucomicrobia	Fusobacteria	Proteobacteria	Chordata	Chordata
Class	Clostridia	Bacteroidia	Actinobacteria	Verrucomicrobiae	Fusobacteria	Gammaproteobacteria	Mammalia	Reptilia
Order	Clostridiales	Bacteroidales	Bifidobacterales	Verrucomicrobiales	Fusobacterales	Enterobacterales	Primates	Squamata
Family	Ruminococcacae	Bacteroidaceae	Bifidobacteriaceae	Akkermansiaceae	Fusobacteriaceae	Enterobacteriaceae	Hominidae	Varanidae
Genus	Faecalibacterium	Bacteroides	Bifidobacterium	Akkermansia	Fusobacterium	Escherichia	Homo	Varanus
Species	prausnitzii	thetaiotaomicron	breve	muciniphila	necrophorum	coli	sapiens	komodoensis

Figure 1. Taxonomic classification of the most abundant bacterial phyla present in the gut.

Subsequently, phyla can be further subdivided at different levels; Class, Order, Family, Genus and (sub) species. From each of the most dominant phyla present in

the gut, an example of a bacterium from phylum to species level is depicted. For comparison also humans and a Komodo dragon, belonging to the same phylum, are displayed in the Animalia kingdom.

Techniques to study the microbiota

The microbiota can be studied by several techniques. In the past, researchers depended highly on culturing techniques. However, many bacteria are very difficult to culture. With recent innovations in the field of sequence technology and analysis, scientists are now able to determine and analyse these difficult to culture bacteria. In the 2010-2020 decade, sequencing of a small part (for instance the V4 region of about 250 nucleotides) of the 16S ribosomal RNA gene, was most commonly used. The 16S rRNA gene consists of around 1500 nucleotides and contains regions conserved among all bacteria and archaea, interspersed with nine regions (V1 to V9) that are highly variable among bacterial phylotypes. Phylotypes are defined as a group of 16S sequences having 97-99 % sequence identity, and usually equals taxonomically to genus or sometimes species level. Because only a small part of the complete genome is sequenced, one can determine the composition and relative abundance of bacterial taxa present in a sample in a relatively fast and inexpensive way. Samples are generally compared using alpha-diversity (within-sample diversity; one value per sample, an example is a Shannon-diversity index) and beta-diversity (between-sample diversity; pair-wise values for all sample combinations, an example is Unifrac-distance or Bray-Curtis-dissimilarity). Bacterial abundance and composition gives insight in 'who is present', which does not necessarily describe the functionality ("what are they doing"). To analyse the functional potential, metagenomic shotgun sequencing is more suitable **[28]**. This technique shotgun sequences the total DNA of the microbiota. Therefore the complete genomic make-up of the microbiota (which is called, the 'metagenome' or more frequently the 'microbiome') is assessed, and one can not only determine the composition, but also predict the potential functions of the microbiota. In *Figure 2*, a general overview of pipelines of 16S and metagenomic shotgun sequencing is depicted.

While with 16S analysis only the 16S rRNA containing bacteria can be studied, metagenomics is not limited to sequencing bacteria, the microbiota including viruses, parasites and fungi, can be analysed. Importantly, analysing the results of sequencing

is challenging and relies on specialized and skilled bioinformatic experts. In addition, metagenomic sequencing is expensive, and one approach to lower the costs is minimisation of sequence depth or coverage. Coverage is the number of unique reads that include a given nucleotide (copy number) in the reconstructed sequence [29]. (Ultra) deep sequencing refers to the general concept of aiming for high copy number, which allows for detection of lowly abundant species or sequence variants in mixed populations. To reduce the amount of data and lower the costs, the sequence depth can be decreased. This is referred to as shallow sequencing [30]. A complete overview of all techniques studying the microbiota with all advantages and limitations is shown in Table 1.

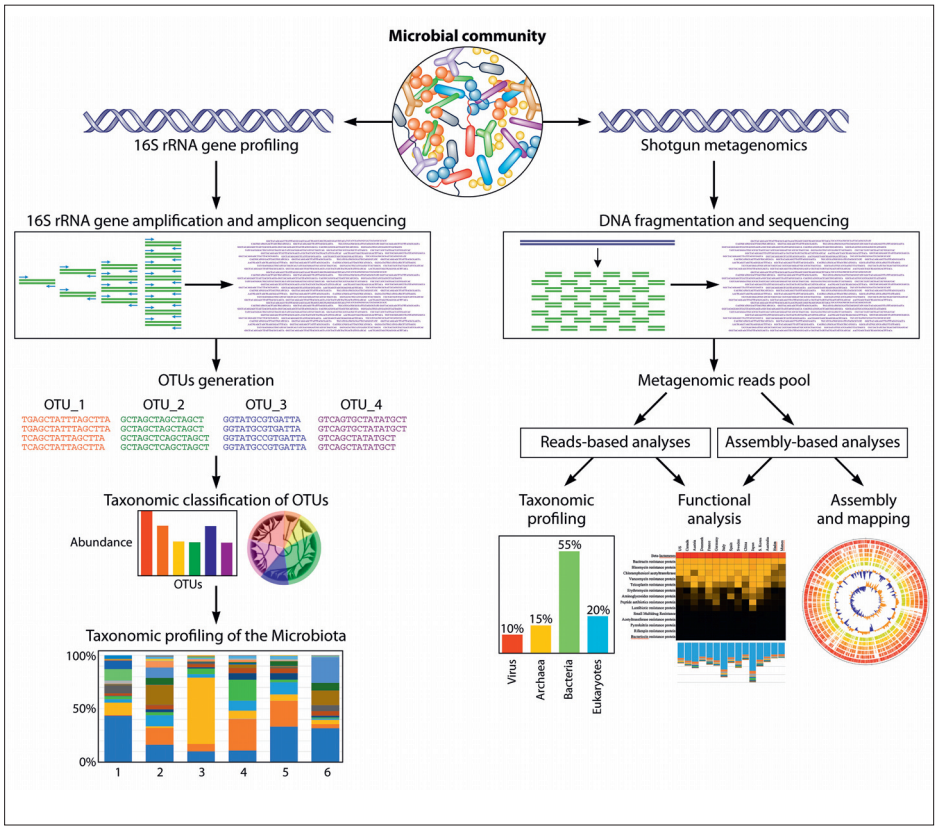

Figure 2. 16S rRNA sequencing and metagenomics pipeline.

Adopted from Milani and co-workers [31].

General overview of the bioinformatic pipelines for the 16S rRNA gene (amplicon) sequencing on the left and shotgun metagenomics on the right. First microbial DNA is extracted and subsequently sequenced. With 16S rRNA analysis, only (part of) the 16S rRNA is sequenced. Highly similar sequences are grouped into Operational Taxonomic Units (OTUs), which for identification can be compared to a 16S database. The microbiota community can be described in terms of which OTUs are present, their relative abundance, and/or their phylogenetic relationships. On the right, metagenomic shotgun sequencing of the microbiota is depicted, in which the total extracted and fragmented DNA is shotgun sequenced. The resulting DNA sequences are either pieced together using assembly algorithms or reference databases, or analysed in an unassembled manner to monitor whole-community functional capabilities. The phylogenetic origins of microorganisms and their functions can be determined by comparison with previously annotated genes in a database.

A famous project trying to unravel the composition and function of the microbiota is the Human Microbiome Project *(Figure 3)*. This project illustrates the value of the use of a combination of different techniques; the difference between 'who is present' is studies with 16S and 'what can they do' with metagenomics. The combination of both techniques revealed that healthy individuals can have a very different microbiota composition, while the relevant functions of those microbiota compositions for their host remained very similar between these individuals **[32]**.

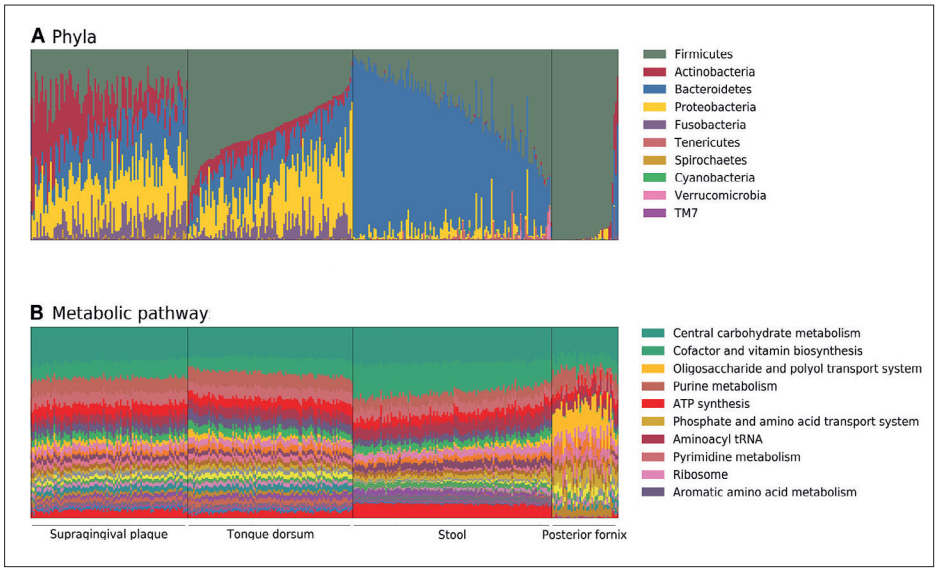

Figure 3. Carriage of microbial taxa in the gut varies while predicted metabolic pathways remain stable within a healthy population.

Adopted from the Human Microbiome Project [32].

Vertical bars represent microbiome samples by body habitat in seven locations with both shotgun and 16S data; bars indicate relative abundances coloured by microbial phyla from binned OTUs (a) and metabolic modules (b). Legend indicated most abundant phyla or pathways by average within one or more body habitats. OTU: operational taxonomical unit.

Measuring the functional capacity of the microbiota with metagenomics does not necessarily provide insight in what the bacteria are actually doing. The following – omics techniques each unravel different layers of information of the microbiota as complex ecosystem *(Figure 4)*. The direct measurement of transcripts (mRNA) with (meta)transcriptomics or proteins with (meta)proteomics is at present increasingly used as complementary technique to metagenomics **[33]**. The combination of metagenomics with metatranscriptomics enables the identification of gene repression or induction under specific conditions, and can additionally distinguish metabolically active from inert or dead micro-organisms **[34]**. Not all transcripts are however translated into proteins. Therefore metaproteomics, in which the expressed proteins are measured with high resolution mass spectrometry, should provide more insight into gut microbial functionality as compared to metatranscriptomics. In addition, it determines proteins derived from the microbiota and their host, which is important when studying microbiota-host interactions. In the past this technique suffered from low measurement depth and lack of efficient bioinformatic tools **[35]**. The availability of new metaproteomic data processing tools has enabled better characterization of the proteome. The fecal metabolome is often regarded as an endpoint read-out of biological processes originated from the gut microbiota and their host. To measure these metabolites, nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry can be applied, known as metabolomics. Identified metabolites in faeces can include those derived from microbiota (e.g. Short Chain Fatty Acids, lipopolysaccharides) or the host (e.g. anti-microbial peptides (AMPs)). Integrating multi-omics data provides a comprehensive overview of microbiota composition, function and metabolomic activity, in relation to its host. Due to the increased complexity and diversity of multi-omics data, efficient bioinformatic tools, advanced statistical methods and machine-learning approaches are needed, which are at present only available in some microbiota expert teams. The multi-omics approach is of particular importance for translational research (microbiome analysis into clinical applications), because chronic human illnesses or diseases associated with a perturbed microbiota are unlikely to be caused by a single bacterium, protein or pathway.

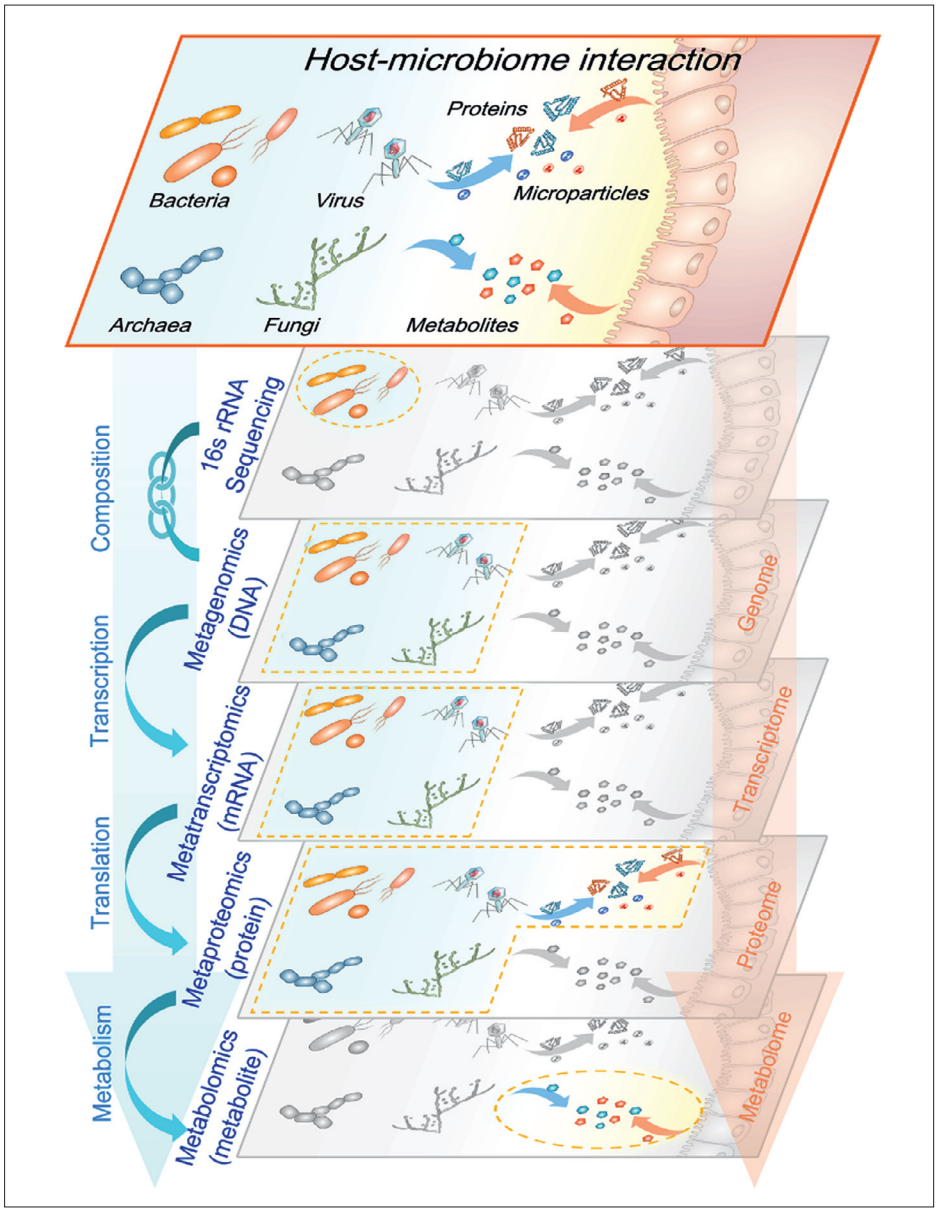

Figure 4. Multi-omics approach of microbiota analysis.

Adopted from Xu Zhang and co-workers [36].

The human gut consists of host and microbial cells, as well as secreted proteins, metabolites, and microparticles, all of which may interact with each other to impact human health. Different meta-omic approaches each examine different aspects of this intestinal ecosystem at different levels with their own advantages and disadvantages.

Molecular techniques to study bacterial strains in depth

To interpret the metagenomic (annotated) data, culturing is still essential. To determine the functions and phenotypes of unknown bacterial genes, culturing of the microbial communities (culturomics) has revived and regained interest [37, 38]. In addition, complete genetical and phenotypical characterisation of cultured isolates is essential to define microbiota interactions (between bacterial species or between bacteria and host) that are difficult or yet impossible to predict unless tested biologically [39].

Once isolated, the complete DNA of a bacteria can be determined with whole genome analysis (WGS). The introduction of next-generation sequencing (NGS) machines had made WGS attainable in terms of costs and time. The generated WGS data can then be subjected to a variety of molecular analyses to characterise the bacterium in terms of antibiotic resistance, molecular epidemiology (e.g. typing) and virulence. Due to its high resolution and inter and intra-reproducibility, WGS is highly suitable as typing method. One means of exploiting WGS data is the identification of single nucleotide polymorphisms (SNPs) that vary among isolates. An alternative approach is multi locus sequence typing (MLST). With common MLST, a limited number of housekeeping genes is sequenced and every sequence variant of a housekeeping gene (locus) is assigned as a distinct allele. For each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST). To increase the resolution and to make optimal use of WGS, many more loci can be employed with core genome MLST (cgMLST) (for *E. coli* around 2500 of total 7700 genes) and whole genome MLST (wgMLST) [40]. An advantage of MLST, cgMLST and wgMLST is that loci used in the schemes are readily conserved and shared among laboratories using online databases. In addition to online (commercial or free available) databases for typing, also many online databases exist to further characterize bacterial isolates with respect to genes or mutations in chromosomes or plasmids associated with resistance, serotypes, plasmids or specific gene functions. A new trend of microbiological characterization is the application of long read sequencing by rapid methods, such as the "MinION" nanopore. Analysis with longer read lengths will alleviate numerous computational challenges surrounding genome assembly as short-read methods can miss some randomly-distributed segments of genomes present in phages, plasmids and virulence factors. Therefore long-read sequencing provides the tool to study the presence and composition of antibiotic resistant genes containing plasmids [41].

Table 1. Techniques to study the microbiota, description, advantages and limitations.

Adapted from **[42]**

Technique	Advantage	Limitation
Marker gene sequencing (16S, ITS)	• Low cost • Relatively quick and easy to manage and interpret • Also suitable for low-biomass and highly host-contaminated samples • Large, available public databases	• No discrimination between dead or alive bacteria • Only determination to Operational Taxonomic Unit (equivalent to genus or sometimes species level) • Amplification bias because of targeted primer (sensitivity and copy number among species differs) • Choice of primers and variable region (V1-V9) magnifies biases • No functional information
Metagenomic analysis	• Determination and relative abundance of microbial functional genes, microbial taxonomic and phylogenetic identity to species and strain level (for known organisms) • Captures the complete microbiota (bacteria, phages, viruses, plasmids, microbial eukaryotes) • Can be mined for novel gene families • Possibility to assemble population-averaged microbial genomes	• Relatively expensive, laborious and complex sample preparation and analysis. • No discrimination between dead or alive bacteria • Contamination from host-derived DNA and organelles may obscure microbial signatures. • Significant proportion of data cannot be functionally assigned due to a lack of close matches in reference databases. In particular with viral data (where over 80% of sequence reads have no known match) • Often difficult to assign function unambiguously based on sequence similarity alone • Can be difficult to assemble genomes, particularly from less abundant members of the microbiota or when a community contains many closely related species. This means that, even if a function can be ascertained, it may be difficult to assign it to specific species within the whole community. • Population-averages microbial genomes tend to be inaccurate owing to assembly artefacts
Metatranscriptomics	• Can estimate which microorganisms in a community are actively transcribing (if paired to marker gene or metagenomic analysis) • Can discriminate between active and alive versus dormant or dead microorganisms or extracellular DNA • Captures dynamic intra-individual variation • Directly evaluates microbial activity, including responses to intervention and event exposure	• Expensive, laborious and complex sample preparation and analysis • Rely on obtaining sufficient high-quality RNA from the sample (challenging due to ubiquitous RNAses in host-derived samples) = fast processing is mandatory • Saturated with less informative, highly abundant transcripts (i.e. ribosomal proteins, major outer membrane proteins) obscuring the detection of functionally important, but less abundant transcripts • Requires paired DNA sequencing to decouple transcription rates from bacterial abundance changes
Metaproteomics	• Can measure which proteins are produced by active members of the microbiota • Measures not only microbiota but also proteins of the host (essential for microbiota – host interactions)	• Expensive and complex data analysis • Lower depth of measurement compared to metagenomics and metatranscriptomics, and can only capture 10-20% of expressed protein. MS spectra can also be saturated with the highly abundant proteins from dominant species, issue likely to be resolved by increasing the speed of time of MS scanning. • Lack of universal guidelines and protocols for proper performance and analysis of metaproteomic experiments
Metabolomics	• Can measure which proteins are produced by active members of the microbiota • Measures not only microbiota but also proteins of the host (essential for microbiota – host interactions)	• Expensive and complex data analysis • Difficulty to distinguish host- and microbiome-origin metabolites and directly link metabolites to specific taxa. Co-variations between metabolites and microbial species not yet known.

Microbiota and colonization resistance

In a healthy state, there is a symbiotic relationship between the host and the microbiota. The microbiota fulfils many functions which are of benefit for the host. Similarly, host factors are required to maintain a balanced microbiota. The gut microbiota contributes to host health by, amongst others degradation of carbohydrates (food), synthesis of bioactive substances (for example vitamins) and colonization resistance against pathogens [43]. Colonization resistance is the mechanism whereby the microbiota protects against colonization of exogenous and often pathogenic microorganisms. The importance of a healthy indigenous intestinal microbiota for the presence of colonization resistance was first recognized in the 1950s, and it was initially referred to as "antibiotic associated susceptibility" [44]. When pre-treated with streptomycin, a minimal infectious dose of only 10 instead of 10^6 *Salmonella enterica* bacteria was sufficient for mice to become infected [45]. Together with the observation that the susceptibly decreased when mice were exposed to coprophagy with normal mouse feces led to the idea that antibiotics cause a perturbation of the gut microbiota [45]. Colonisation resistance is the result of direct or indirect factors. Direct colonisation resistance refers to the direct suppression of intestinal pathogens by competitive exclusion (competition for nutritional niches or space) and by antimicrobial activities like bacteriocins [46]. For instance, *Bacteroides thetaiotaomicron* consumes carbohydrates used by *Citrobacter rodentium*, a gastro-enteritis pathogen in mice [47]. *Bacteroides thuringiensis* secretes thuricin, a bacteriocin (small-spectrum antibiotic) that directly targets spore-forming Bacilli and Clostridia, including *Clostridium difficile* [48]. In addition, commensal bacteria can also indirectly control invading pathogens by enhancing host immunity and mucus production in the intestines. The microbiota plays an important role in the development, training and maintaining of the immune system [49]. An example of this has been observed with *B. thetaiotaomicron* which can induce the host to produce antimicrobial C-type lectins that target Gram positive bacteria [50].

Gut microbiota and disease

The significance and role of many bacterial species in health and disease are poorly understood, but it has become evident that the gut microbiota is disturbed in a wide

range of diseases. This perturbation in function and composition of the microbiota is called dysbiosis, which is still difficult to distinguish from homeostasis or healthy microbiota, because a healthy reference or core microbiota is not (yet) defined. Importantly, dysbiosis is not only associated with intestinal disorders, such as inflammatory bowel disease (IBD; morbus Crohn and ulcerative colitis) **[51-53]** and irritable bowel syndrome (IBS) **[54, 55]**, but also with a wide range of extra intestinal conditions, such as metabolic syndrome **[56-58]**, (non)-alcoholic fatty liver disease (NAFLD) **[59-62]**. Neurological diseases like Parkinson's disease and multiple sclerosis, and psychiatric disorders are also considered to be associated with intestinal dysbiosis via the so-called hypothetical gut-brain axis **[63-66]**. Whether the microbiota is truly involved in the pathogenesis of those disorders awaits to be seen, but for many diseases, a role in the development or course of the disease has been shown in animal models **[9]**. Interestingly, the microbiota is not only involved in the pathogenesis, but also alters the pharmacokinetics or may mediate (side effects of) certain drugs direct or via CYP-like proteins **[67, 68]**. The microbiota is therefore, of importance in drug discovery, risk assessment and dosing regiments for various infectious and non-infectious diseases.

It is foreseen that all above mentioned diseases have a disease-specific profile of dysbiosis, although a conclusive description of dysbiosis in specific disorders is still lacking. In general, dysbiosis is characterized by a reduced diversity of the microbiota, with a reduction of certain species of the normally abundant Firmicutes or Bacteroidetes phylum (such as Clostridium cluster IV an XIVa, *Faecalibacterium prausnitzii*, *Akkermansia*, *Eubacterium halli*) and a higher abundance of the normally less abundant (opportunistic) Proteobacteria (like *Escherichia coli* or *Klebsiella* species). Whether such a perturbed microbiota is involved (driving or maintaining) in the pathogenesis or an epiphenomena (a consequence of the disease) is not yet elucidated for many diseases. In this regard, *Clostridioides difficile* infection appears unique, as dysbiosis of the microbiota is mandatory. Infection with *C. difficile* represents the classic example of a disease that is caused by a dysbiotic microbiota, providing a model to study the dysbiotic microbiota and interventions targeting dysbiosis.

Clostridioides difficile infection as result of intestinal dysbiosis

Introduction & pathogenesis

Clostridioides difficile is a Gram-positive, spore forming, obligate anaerobic bacterium that was identified as part of the normal gut flora of healthy infants in 1935 [69]. The species name is derived from the initial difficulties to culture and identify *C. difficile*. The genus name was used for more than 80 years, but recently, based on phenotypic, chemotaxonomic and phylogenetic analyses, a novel genus *Clostridioides* gen. nov. has been proposed for *Clostridium difficile* as *Clostridioides difficile* [70]. Fortunately, the abbreviation remained intact. *C. difficile* is considered as part of the gut commensal microbiota of both humans as well as animals and is transmitted by spores via the fecal-oral route. Most vegetative *C. difficile* bacteria are killed in the stomach [71]. *C. difficile* spores are however acid resistant and will subsequently pass the stomach. After germination of the spores in the small intestine under the influence of bile salts, vegetative bacteria enter the colon where they can remain inactive (asymptomatic colonization) or cause an infection (CDI, *C. difficile* infection) varying from self-limiting and mild diarrhoea to life-threatening pseudomembranous colitis *(Figure 5)* [72]. Several virulence factors, including flagella and hydrolytic enzymes have been associated with disease [73].

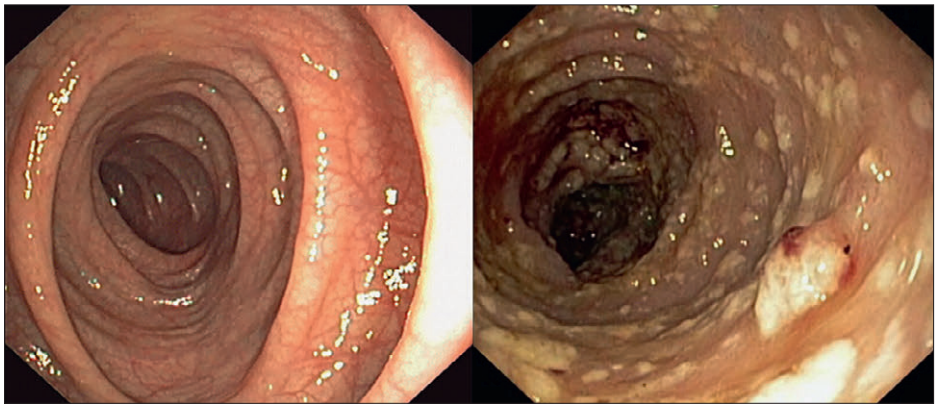

Figure 5. Healthy colon mucosa (left) versus Pseudomembranous colitis due to a *Clostridioides difficile* infection (right).

Adapted from Terveer and colleagues [74].

The two most important virulence factors of *C. difficile* are the exotoxins that are produced, toxin A and B. Both toxins are cytotoxic for a number of different cell types, increase vascular permeability by opening tight junctions between cells, and cause apoptotic cell death [72,73]. In addition, the toxins induce an inflammatory response mediated by tumour necrosis factor-alpha (TNF-α) and pro-inflammatory cytokines which contribute to the characteristic formation of pseudo-membranes [73].

Colonization versus infection

C. difficile infection (CDI), previously described as *C. difficile*-associated diarrhoea (CDAD), is the most common cause of healthcare associated diarrhoea in Western countries [75]. By shedding of spores from symptomatic patients, *C. difficile* can spread within healthcare facilities, which can subsequently result in new symptomatic patients and eventually clusters or outbreaks. Not all newly diagnosed CDI patients can be linked to other symptomatic CDI cases. Two studies using multi-locus sequence typing and whole genome sequencing with single-nucleotide typing respectively, could only link 25 %-40 % of CDI patients to a previously identified CDI patient [76,77]. Recent studies show that also asymptomatically *C. difficile* colonized patients contribute to the spread of *C. difficile* spores via healthcare workers or the environment [78-80]. Riggs showed that *C. difficile* spores were present on the skin of asymptomatic carriers and easily transferred to investigator's hands and the environment [80]. Although transmission events from asymptomatic carriers as index patient remains rare [79], asymptomatic carriers may still importantly contribute to transmission, as they likely outnumber symptomatic CDI patients. In addition, the *C. difficile* carriers have themselves a higher risk of progression to CDI [81-83]. Approximately 5 % (0-15 %) of healthy individuals are asymptomatically colonized with *C. difficile* [82]. The incidence increases during prolonged hospitalization to 4 - 21%, and in nursing home residents colonization rates of 4 – 10 %, but up to 51 %, have been reported [82]. These observations prompted to survey *C. difficile* colonization rates in the healthy population and amongst nursing home residents in the Netherlands. Asymptomatic carriership is usually undetected, as routine screening is not performed. Interestingly, in Canadian tertiary institution in Quebec City, isolation precautions for colonized patients identified upon admission, decreased healthcare associated CDI [84], suggesting that asymptomatic *C. difficile* carriers significantly contribute to spread of CDI.

Microbiological diagnosis of *Clostridioides difficile*

The diagnosis of CDI is not always easy and straightforward. Because of the possibility of asymptomatic colonization and the potential presence of other causes for diarrhoea, detecting the presence of *C. difficile* in the feces does not necessarily defines disease. The main problem is absence of a fast and easy to perform test that differentiates between colonization and infection with high positive and negative predictive value. Of note, the presence of two gold standards, each with their own benefits and drawbacks also complicates the interpretation of research in this area. The cell cytotoxicity assay (CCNA) as gold standard test, detects the presence of free *C. difficile* toxin by a cytopathic effect in cell culture that is neutralized by the presence of antitoxin [85]. Detection of free toxin in the stool correlates best with CDI symptoms [86], however is labour intensive and non-standardized [86]. Toxigenic culture (TC), the second gold standard, evaluates the potency of cultured isolates to produce toxins in vitro [86, 87]. TC is considered the most sensitive of both assays, however in return less specific as asymptomatically colonized individuals are also tested positive. Given these drawbacks, one can question whether a true gold standard exists at all. Due to its labour intensiveness and required expertise, the availability of both tests in routine clinical microbiology laboratories is limited. Furthermore, the test results are only available after several days, often too late for clinical decision making. In Table 2, estimates of pooled sensitivity and specificity for different diagnostic CDI tests compared to the gold standard are shown. These were used to calculate positive and negative predictive values (PPV and NPV) for the different tests at varying hypothetical CDI prevalence's, depicted in Table 3. The most rapid, and easy to perform diagnostic tests are toxin enzyme immunoassays (EIAs), but it has become evident that these assays lack sensitivity to accurately diagnose CDI (Table 2), especially in a low prevalence disease setting ranging between 5-10 % (Table 3) [88]. In contrast a glutamate dehydrogenase (GDH) EIA or toxin nucleic acid amplification test (NAATs) display high sensitivity, but also lack specificity. The European diagnostic guidance document advises therefore a two-stage algorithm, using a NAAT or GDH EIA as sensitive screening assay, in combination with tests to detect the presence of free toxins in stools as marker of disease activity [88]. Using this guidance document, the European Center for Disease Prevention and Control (ECDC), formulated a practical advice to apply the two-step algorithm in studies of CDI (Table 4) [89].

Table 2. Pooled sensitivities and specificities of categories of tests

Type	Compared to CCNA			Compared to TC			Compared to culture		
	N studies	Sensitivity (95% CI)	Specificity (95% CI)	N studies	Sensitivity (95% CI)	Specificity (95% CI)	N studies	Sensitivity (95% CI)	Specificity (95% CI)
EIA GDH									
total	12	0.94 (0.89-0.97)	0.90 (0.88-0.92)	8	0.96 (0.86-0.99)	0.96 (0.91-0.98)	11	0.94 (0.86-0.97)	0.96 (0.92-0.98)
well-type	5	0.94 (0.91-0.97)	0.92 (0.92-0.93)	1	0.94 (0.93-0.96)	0.94 (0.94-0.95)	4	0.89 (0.86-0.91)	0.91 (0.90-0.92)
membrane-type	7	0.98 (0.78-1.00)	0.90 (0.87-0.93)	7	0.97 (0.84-1.00)	0.96 (0.90-0.99)	7	0.93 (0.84-0.97)	0.98 (0.95-0.99)
EIA Tox A/B									
total	27	0.83 (0.76-0.88)	0.99 (0.98-0.99)	29	0.57 (0.51-0.63)	0.99 (0.98-0.99)			
well-type	18	0.85 (0.77-0.91)	0.98 (0.96-0.99)	16	0.60 (0.52-0.68)	0.98 (0.97-0.99)			
membrane-type	9	0.79 (0.66-0.88)	0.99 (0.98-0.99)	13	0.53 (0.45-0.61)	0.99 (0.97-1.00)			
NAAT	14	0.96 (0.93-0.98)	0.94 (0.93-0.95)	32	0.95 (0.92-0.97)	0.98 (0.97-0.99)			

CI: confidence interval
CCNA: cel cytotoxicity neutralization assay
EIA: enzyme immunoassay
GDH: glutamate dehydrogenase
NAAT: nucleic acid amplification test
TC: toxigenic culture

Table 3. PPV and NPV for different categories of index tests at hypothetical CDI prevalence's of 5, 10, 20 and 50%

Type test	CDI prevalence 5%		CDI prevalence 10%		CDI prevalence 20%		CDI prevalence 50%	
	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV
Well-type EIA GDH	38	100	54	99	72	98	91	94
Membrane type EIA GDH	34	100	52	100	71	99	91	98
Well-type EIA tox A/B	69	99	83	98	91	96	98	87
Membrane type EIA tox A/B	81	99	90	98	95	95	99	83
NAAT	46	100	64	100	80	99	94	96

Pooled estimates of sensitivity and specificity compared to cell cytotoxicity neutralization assay were used to calculate the predictive values.
CDI: *Clostridium dificile* infection
EIA: enzyme immunoassay
GDH: glutamate dehydrogenase
NAAT: nucleic acid amplification test
NPV: negative predictive value
PPV: positive predictive value

Table 4. Practical advice to apply the two-step algorithm by the ECDC

Categorization of CDI diagnosis	CDI diagnostic algorithm		
	Screening test	**Confirmatory test**	**Optional third test**
ESCMID-recommended	NAAT	Toxin A/B EIA	N/A
	GDH EIA	Toxin A/B EIA	NAAT or toxigenic culture
	GDH and Tox A/B EIA	NAAT or toxigenic culture*	N/A
Not recommended	All other algorithms		

CDI: Clostridium difficile infection
ESCMID: European Society for Medical Microbiology and Infectious Diseases
NAAT: Nucleic acid amplification test
Toxin A/B EIA: enzyme immunoassay that test for both toxins A and B
GDH: glutamate dehydrogenase
N/A: not applicable
* In this testing strategy, NAAT or toxigenic culture is an optional second test (there is no third test option)

Gut microbiota & *Clostridioides difficile* infection

The propensity of *C. difficile* spores to colonize the intestinal tract and subsequently outgrow and produce toxins, is highly influenced by the host microbiota and metabolome. In healthy individuals, the immune system along with a complex interplay of the gut microbiota, by competition for food and place, excretion of metabolites and bacteriocins, suppresses the growth of *C. difficile*. This defence mechanism is also known as colonization resistance [90]. Upon disruption of the microbiota due to exogenous factors such as antimicrobials or other medication as proton pump inhibitors or chemotherapy, colonization resistance decreases and *C. difficile* can proliferate, produce toxins and cause disease [24, 91]. Of the antibiotics, clindamycin, fluoroquinolones and cephalosporins, monobactams and carbapenems are in particular notorious for serious microbiota disruption [92]. By disrupting the microbiota, antibiotics have a selective effect on several key factors of the microbiota to suppress *C. difficile*. For example, microbiota changes can inhibit conversion of the primary bile acids with *C. difficile* spore-germinating capacity, to the *C. difficile* inhibiting secondary bile acids, enabling the outgrowth of *C. difficile* spores [93, 94]. These disruptions of the microbiota and consequent vulnerability of disease progression are more common in the fragile elderly population. The precise microbes responsible for inhibition or progression from *C. difficile* colonisation to infection have not been identified. However,

few suggestions have been made [95]. For example, some bacteria, like for instance *C. scindens*, convert the *C. difficile* enhancing primary bile acids to the inhibiting secondary bile acids [93, 94]. Moreover, several studies have reported the recovery of Bacteroidetes and members of the Firmicutes phylum; the families Lachnospiraceae (formerly known as Clostridium cluster XIVa) and Ruminococcaceae (formerly known as Clostridium cluster IV), including *Faecalibacterium prausnitzii, Eubacterium rectale*, *Roseburia intestinalis* and other known butyrate-producing bacteria along with successful clinical recovery from CDI [96-99]. Additionally, the level of Bacilli and Proteobacteria, generally found at high levels in patients with CDI, decreased after successful recovery [98]. Those observations will guide the future development of bacterial mixtures to prevent and treat CDI. Finally, the involvement of host immunity in the gut microbiota-mediated colonization resistance to CDI is incompletely understood but has recently been studied in mice, suggesting that IL-22-mediated host glycosylation stimulates the growth of commensal bacteria that compete with *C. difficile* for the nutritional niche [100].

Treatment of *Clostridioides difficile* infection

In 2014, the CDI treatment guideline of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) advised metronidazole, vancomycin and to a lesser extent fidaxomicin as the cornerstones of the CDI treatment [101]. However, after publication of this guideline, a large multicentre RCT showed that metronidazole is inferior to vancomycin in the treatment of both severe and non-severe CDI [102], with cure rates of respectively 81% versus 73%. The lower cure rate could be partly explained by the poor intestinal concentration of metronidazole in the lower gastrointestinal tract [103]. As result, metronidazole is currently replaced by vancomycin in most guidelines as first line CDI therapy [104]. Similarly, the IDSA guidance document recommended either vancomycin or fidaxomicin over metronidazole for an initial episode of CDI [105]. After treatment of an initial episode of CDI, recurrence occurs within eight weeks in 15-25% [102, 106, 107]. For a patient with one or two recurrences, the risk of further recurrences is increased to 40-65% [106, 108]. Fidaxomicin seems evenly effective as vancomycin in curing the symptoms of a first CDI episode, though due to its small antibiotic spectrum, relapses occur significantly less, 25.3% versus 15.4% respectively [102, 109, 110]. In addition, in patients with a first relapse, less second

relapses occurred in the fidaxomicin treated versus a vancomycin treated group (19.7 % versus 35.5 %) [107]. A variation of the 10 days fidaxomicin treatment is a so-called extended-pulsed fidaxomicin (200 mg oral tablets, twice daily on days 1-5, then once daily on alternate days on days 7-25). Extended-pulsed fidaxomicin was superior to standard-dose vancomycin for sustained cure of *C. difficile* infection with a cure rate of 70 % versus 59 % at 30 days after end of treatment [111]. Although the initial treatment costs of (extended-pulsed) fidaxomicin are very high (€ 1680,68 2dd200mg 10 days), it may be cost-effective as first line therapy in older patients, in comparison to vancomycin (€ 410.73 4dd250mg, 10 days), due its increased efficacy [111, 112]. Nevertheless, due to the high costs, fidaxomicin is mainly prescribed for patients with recurrent CDI in the Netherlands. A new, interesting treatment strategy is provided by bezlotoxumab, an anti-*C. difficile* monoclonal antibody, which can be prescribed as additive to standard antibiotic therapy. In a large phase 3 study, bezlotoxumab significantly lowered the rate of recurrence within 12 weeks in comparison to standard therapy (17 % versus 27 %) [113]. As for fidaxomicin, the current price of bezlotoxumab in combination with the limited additional beneficial effects hampers its broad scale implementation in clinical practice. Despite high expectations, treatment approaches directed to bind or neutralize *C. difficile* toxin in the intestinal tract, were not successful. In most cases, the design of the studies was not optimal or insufficient number of patients were included. Tolevamer, a *C. difficile* toxin binding polymer, was inferior to antibiotic treatment in two RCTs comparing tolevamer with vancomycin or metronidazole [102]. Unfortunately, tolevamer was not tested in combination with anti-CDI antibiotics. Several attempts have been made to develop immune whey with anti-CDI antibodies obtained by vaccination of cows. Though in vitro studies were promising, only one clinical trial was started but stopped due to bankrupt of the company [114, 115].

Despite of the above described treatment modalities, a subgroup of patients suffers from persisting CDI, with continuing relapses after cessation of antibiotics. Recurrent CDI is characterized by a permanently disturbed microbiota, enabling the outgrowth *C. difficile* spores once the anti-CDI antibiotic is stopped. Modifying the gut microbiota to break this cycle and prevents relapses to occur. Of all gut modifying therapies, Faecal microbiota transplantation (FMT) shows at present the best cure rates of over 85 % [96, 116, 117]. In Table 5, FMT and other microbiota modifying therapies are discussed.

Table 5. Overview of gut microbiota modifying therapies for treatment of recurrent CDI

Gut modifying therapy	Comment	Evidence in CDI treatment
Prebiotics	Dietary components that foster the growth of beneficial micro-organisms and stimulate a healthy microbiota. For instance, fibres of which Short Chain Fatty Acids (SCFA) are produced, which are healthy for the host.	An RCT with oligofructose amongst 132 patients showed that a relapse of diarrhoea occurred in 8.3% of oligofructose treated patients versus 34.3% of placebo treated patients [118]
Probiotics	A live microbial feed supplement which could be of benefit for the host. Limited efficacy. One bacterial mix beneficial for all conditions seems too simple. Generalizability of the results is challenging due to varied probiotic preparations in the research. Examples of studied probiotics: *Saccharomyces boulardii*, *Lactobacillus plantarum*, non-toxigenic *C. difficile* strain, a multi-strain preparation of Lactobacilli and Bifidobacteria.	Systematic review of RCTs: Two studies have found benefit to probiotics in prevention of rCDI: *S. boulardii* [119, 120] and a non-toxigenic *C. difficile* strain M3 [121] when compared to placebo. Cochrane systematic review (n=8672, 31 RCTs). Moderate certainty evidence: probiotics effective to prevent CDAD (NNTB = 42 patients, 95% CI 32-58). Post hoc subgroup analysis: probiotics effective with CDAD baseline risk >5% (NNTB = 12), but when baseline risk ≤5%. When probiotics administered together with non-CDI antibiotics RR reduction of 0.4 in acquiring CDI (1.5 vs 4%), NNT 40 [122].
Synbiotics	Combination of pre- and probiotics, for instance yoghurt containing *Lactobacillus sp.*	No
Defined bacterial consortium	Bacterial mix of healthy bacteria	Poof of principal Phase I study with bacterial spores (approximately 50, SER-109), effective in subset of patients [123]. However, failure to treat rCDI in phase II study. Multiple studies with rational selected bacterial consortia (VE303, Vedanta) underway.
Phage therapy	Transfer of bacteriophages	Proof-of-principle: Faecal filtrate (including meta-bolites/bacteriocins) was proved to cure (n=5) CDI patients [124]. In addition, FMTs with increased bacteriophage α-diversity were more likely to successfully treat rCDI [125]
Faecal microbiota transplantation	Transfer of complete healthy microbiota ecosystem to diseased microbiota, containing living bacteria, bacteriophages, metabolites and bacteriocins.	Established to prevent relapses in multiple, recurrent CDI.
Metabolites (postbiotics)	Transfer of beneficial microbial products that prevent germination, colonization and/or toxin production of *C. difficile*. Without risk of permanent engraftment of potential microorganism with risk, although question whether response permanent and chronic therapy needed.	Ursodeoxycholic acid (UDCA) prevented rCDI in 16 high-risk patients [126].

CDAD: *Clostridioides difficile*-associated diarrhoea

NNTB: number needed to treat to benefit one person

RR: relative risk

Faecal microbiota transplantation, a highly effective microbiota modulating therapy

Recurrent CDI is associated with an impaired immune response to *C. difficile* toxins and more importantly, with a persistent and severely perturbed colonic microbiota [127]. 16S rRNA gene sequence analysis of patients with recurrent disease showed a highly variable bacterial composition in comparison with the normal predominance of Bacteroidetes and Firmicutes in healthy individuals [98, 127]. Furthermore, patients with rCDI showed lower species richness compared with patients with an initial CDI episode or control subjects [97, 127]. This perturbed and diminished microbiota is essential in maintaining the disease, which is supported by the observation that replenishing the microbiota by Faecal Microbiota Transplantation (FMT) results in prompt resolution of rCDI.

The use of human stool as therapy for (mainly) gastrointestinal disorders, such as food poisoning and diarrhoea, was first reported in ancient China [128]. In the fourth century, Ge Hong orally administered faecal suspensions to treat severe diarrhoea, later referred to such suspensions as 'yellow soup" [128]. In 1958, Western literature described the first patients with severe antibiotic-induced colitis who were successfully treated with donor stool containing enemas [42]. With the increased CDI incidence in the 21st century, this 'ancient' therapy regained interest. In 2013, the first randomized trial using FMT to treat recurrent CDI demonstrated a remarkable efficacy in comparison to vancomycin [96]. FMT was successful in 81% of rCDI patients after just one FMT infusion, 94% after multiple infusions, while vancomycin was successful in only 31% of patients [96]. This high efficacy was confirmed in many independent studies that followed [116, 117, 129], and FMT is now advised in guidelines for treatment or recurrent CDI [104, 105]. A meta-analysis by Quraishi, including seven RCTs and 30 case series, showed that FMT was more effective than vancomycin in resolving recurrent and refractory CDI with a relative risk of 0.23 and a clinical resolution of 92% [116]. The second meta-analysis by Moayyedi, included ten RCTs with a total of 657 patients with *C. difficile*-associated diarrhoea and demonstrated that FMT was significantly more effective compared with placebo or vancomycin treatment, with a relative risk of 0.41 [117]. After FMT, patients show an increase in microbiota diversity, reaching levels that are observed in healthy donors [96, 98]. In conclusion, FMT is a highly effective treatment for patients suffering from multiple recurrent CDI [105].

Table 6. Overview of the outcome of FMT studies performed in patient with various conditions

Disorder	Type of study (references)	Outcome	Comments and important unresolved questions
Recurrent CDI	RCT [96, 129, 133, 134] Meta-analysis [116]	Highly effective, cure rate single infusion >80%	Advised in guidelines for recurrent rCDI [101, 105]
Severe CDI	Case series [135-138]	Effective and safe. An FMT program significantly decreases CDI-related mortality (9.1% versus 21.3%) and colectomy (2.7% versus 6.8%) in patients with fulminant CDI.	May be lifesaving. Probably sequential FMT infusion needed [139]
UC	RCT [140-143] Meta-analysis [53, 144, 145]	Pooled response rate of 29% for achieving endoscopic remission	Optimization of protocol required: Is rational selection of donors required? Is it possible to select patients who are more likely to respond? Should FMT be offered as induction or maintenance treatment? Is pre-treatment (with IBD medication) needed?
CD	Cohort studies [146-148] RCT [149] Meta-analysis [53]	Pooled clinical response rate of 53%. No endoscopic remission achieved	In very small RCT no effect, larger RCTs and rational donor selection needed
IBS	RCT [150-152] Meta-analysis [153, 154]	Large variation in treatment, placebo (auto-FMT/water) and definition and follow-up of treatment effect. According to meta-analysis of 5 RCTs no significant improvement in IBS symptoms with FMT (50% assigned to FMT responded, 56 assigned to placebo responded. In recent RCT with donor selection high response rates: 23.6%, 76.9% ($p<0.0001$) and 89.1% ($p<0.0001$) of the patients who received placebo, 30 g FMT and 60 g FMT, respectively [151]	Larger RCTs needed Which patients may benefit? Is repeated FMT required? How should patients be pre-treated before FMT? Rational donor selection needed?
HE	RCT [155]	Safe, no SAEs related to FMT, no new episodes of HE 150 days post-FMT	Confirmative study needed Rational donor selection needed
MDRO	Cohort studies [156-159] RCT [160]	Suggestive of some effectivity eradicating VRE and ESBL bacteria	Rational donor selection needed. Larger RCT needed with sufficient number of patients
Metabolic syndrome	RCT [161-163]	No effect on clinical endpoints. Transient increased insulin sensitivity	Strictly experimental, first results do not seem promising
Autism	Open-label trial [164]	Effect noted on psychiatric and GI symptoms	Underpowered. Further studies are needed
GvHD	Case series [165-167]	Steroid-refractory GVHD: decreased symptoms. Higher progression-free survival.	Seems safe Underpowered. RCTs are needed

Abbreviations: CD, Crohn's disease; CDI, Clostridioides/Clostridium difficile infection; ESBL, extended-spectrum beta-lactamases; FMT, faecal microbiota transplant; GI, gastrointestinal; GVHD, graft-versus-host disease; HE, hepatic encephalopathy; IBS, irritable bowel syndrome; MDRO, multidrug resistant organism; RCT, randomized controlled trial; SAE, serious adverse event; UC, ulcerative colitis; VRE, vancomycin-resistant enterococci. NAFLD: Non-alcoholic Fatty Liver Disease. Based on van Ooijevaar and colleagues [132]

Large heterogeneity exists among the included studies with respect to donor faeces volume, FMT preparations, route of administration, pre-treatment and numbers of FMTs **[116, 117, 130]**. This underlines the need for standardization of FMT to facilitate FMT, and increase the safety of this new treatment modality, stool banks such as the Netherlands Donor Feces Bank (NDFB) are emerging to standardize and centralize the process of donor selection and screening and to provide ready-to-use donor faeces suspensions to treating physicians. In addition, initiatives are undertaken to further standardize the process of FMT in Europe.

For now, CDI remains the prime disease for which there is a consistent body of evidence supporting treatment by FMT. However our growing understanding of the gut microbiota in health and disease suggests FMT, or more precisely; the concept of modulating the gut microbiota, could have great potential in treating other diseases than CDI **[131, 132]** *(see Table 6)*.

Colonisation with multidrug resistant organisms; unknown association with intestinal dysbiosis

Antibiotic resistance - Introduction

Antibiotic resistance, the ability of a bacterium to resist the action of one or more antibiotics, threatens effective prevention and treatment of infections, and is considered a major threat to public health worldwide **[4, 168]**. Bacterial resistance of several antibiotic classes is nowadays also becoming increasingly more common in the Netherlands **[169, 170]**. Infections with multidrug resistant organisms (MDROs) are not only more difficult to treat, but are accompanied with a rise in health care costs, patient morbidity and mortality **[171]**. Data based on the European Antimicrobial Resistance Surveillance Network (EARS-Net) show that each year, more than 670,000 infections occur in the European Union with antibiotic resistant bacteria, of which 64 % health care associated. Over 33,000 patients die annually as a direct consequence thereof **[172]**. The related cost to the healthcare system is around 1.1 billion Euro's **[173]**.

The Centers for Disease Control and Prevention (CDC) has outlined and prioritized the threats posed by specific MDROs of which drug resistant *Neisseria gonorrhoeae*, carbapenem resistant Enterobacterales (CRE) and *C. difficile* were the most urgent [5]. Although resistance is considerably more common in the Netherlands than 20 years ago, resistance rates are much lower compared to many other European countries [169]. This is partly because of the limited antibiotic use, both in the community as well as in the hospital [1]. Nevertheless, much effort is put in maintaining this low prevalence rate of MDRO. Low prevalence rates give the opportunity to combat resistance, for example with an active search-and-destroy (decolonisation) policy regarding "Methicillin Resistant *Staphylococcus Aureus* (MRSA)". With support of medical microbiologists, infection prevention workers and infectious disease specialists, only 1.2 % of *Staphylococcus aureus* isolates cultured from infections was resistant against methicillin in the Netherlands in 2018 [169]. In contrast, infections with Extended Spectrum β-lactamase-producing (ESBL) Enterobacterales (previously known as Enterobacteriaceae [174]) are much more frequently encountered, both in healthcare facilities and in the community [175] *(Figure 6)*. In the Netherlands, Enterobacterales resistant to both fluoroquinolones and aminoglycosides, are also considered a MDRO,

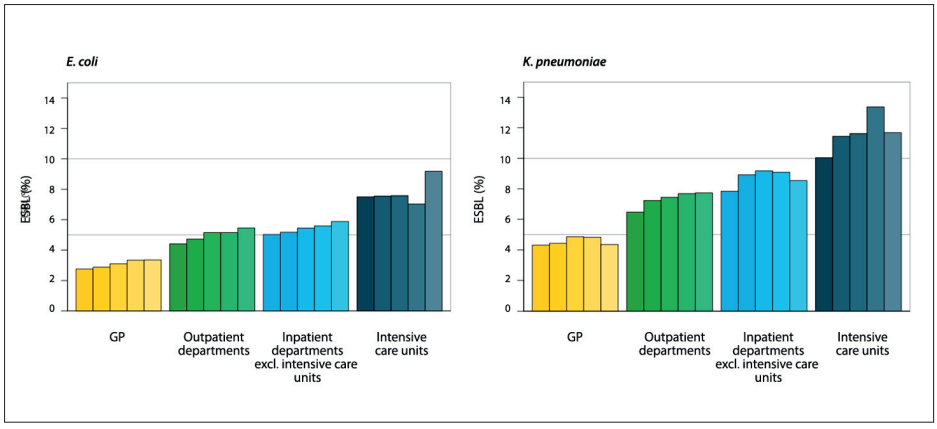

Figure 6. Trend in ESBL producing *E. coli* (left) and K. pneumoniae (right) in the Netherlands in different patient populations (from left to right 2015 to 2019). Isolates of patients attending the general practitioner are depicted in yellow, the outer patient clinic in green, the hospitalised patients (minus intensive care) in light blue and intensive care in dark blue. Adopted from Nethmap [170].

as these antibiotics are important antibiotics used in hospital settings [170, 176]. Of infections with Enterobacterales, urinary tract infections constitute the main clinical syndrome, followed by bloodstream infections with often a urinary or biliary tract origin. On the other hand, similar as *C. difficile*, also MDR Enterobacterales can asymptomatically reside in the gut. The intestinal tract is considered as an important reservoir of human Enterobacterales colonization and infections [177, 178].

Antibiotic resistance - General mechanism of action

Antimicrobial resistance is ancient and is the result of a million years of microbial co-evolution [179]. Most antimicrobial compounds are (derived) from microorganisms. The co-resident target microorganisms have therefore evolved mechanisms to overcome the antimicrobial action, referred to as 'intrinsic resistance'. Intrinsically resistant bacteria are however not the focus of the resistance problem. In contrast to 'acquired resistance', in which a bacterium that was originally susceptible to the antimicrobial compound, gained resistance. There are several major resistance mechanisms, namely 1. Destruction or modification of antibiotic molecule (for instance β-lactamases) 2. Modifications of antimicrobial target and/or binding place (for instance, alteration of Penicillin Binding Protein) 3. Prevention to reach the antimicrobial target by actively extruding by efflux-pumps or decreasing penetration (for instance by porins) or upregulation of the target 4. By-pass of target molecule by microorganism by change in metabolic pathway (for instance some sulphonamide resistant bacteria switch to using preformed folic acid) [180]. Development of acquired resistance can occur 'de novo' or by acquisition of exogeneous resistance genes. Bacteria acquire external DNA through three strategies 1. Transformation (incorporation of naked DNA), 2. Transduction (phage mediated) and 3. Conjugation (bacterial sex). Emergence of resistance in the hospital environment often involves conjugation, a very efficient method of gene transfer that involves cell-to-cell contact and is likely to occur at high rates in the gut microbiota under antibiotic treatment [181]. This is referred to as horizontal spread. Conjugation makes use of mobile genetic elements as vehicles to share valuable genetic information. The most important mobile elements are plasmids and transposons [181].

Antibiotic resistance to cephalosporins by "Extended Spectrum β-lactamases (ESBLs)"

β-lactam agents such as penicillin's, cephalosporins, monobactams and carbapenems, are the most frequently prescribed antibiotics. β-lactamases are bacterial enzymes that inactivate β-lactam antibiotics by hydrolysis and are the predominant mechanism of resistance in Gram-negative bacteria [180]. Extended-spectrum β-lactamase-(ESBL) producing bacteria have the ability to hydrolyse oxyimino-cephalosporins, and monobactams, but not cephamycin's or carbapenems. ESBLs were first described in 1983 and emerged especially in *Klebsiella pneumoniae* and *Escherichia coli* [182]. The genes encoding ESBL resistance, are frequently located on plasmids. Large plasmids carrying both ESBLs and several other resistance genes (e.g. aminoglycosides and fluoroquinolones) are frequently present in the Enterobacterales family [183]. Plasmid-mediated spread of ESBL and other antibiotic resistance genes (ARGs) contributes to the global spread of resistance against many frequently used antibiotics. The global pooled prevalence of ESBL colonization in the intestinal tract of asymptomatic individuals is 14 %, with an increasing trend of 5.4 % annually [2]. Driving factors for this rise are the globalization and the pandemic spread of CTX-M as most dominant ESBL enzyme [184], both in the hospital as well as in the community [2, 175]. CTX-M originated from chromosomal β-lactamases genes from different *Kluyvera* species [185]. *Kluyvera* spp. are ubiquitous found in the environment [186], and the probable environmental reservoir of the resistance genes. These β-lactamases genes were captured and mobilized on a variety of mobile genetic elements mediating rapid dissemination [187]. In the Netherlands, approximately 4.5-8.6 % of the healthy individuals is asymptomatically colonized with ESBL-producing Enterobacterales [188-191]. The prevalence of ESBL colonization varies largely per population; geographically as well as in diseased versus healthy individuals. Travel, kitchen hygiene (for instance not changing the kitchen towel each day) and antibiotic use are important risk factors for carriership of ESBL producing *E. coli* or *K. pneumoniae* in healthy individuals [192-194]. Carriership showed even a seasonal variation, that could not be explained by travel and antibiotic use [193]. Spontaneous clearance of the ESBL varies per bacterial species and per ESBL enzyme. One study reports a long duration of carriage in patients with a clinical infection, since 43 % remained ESBL positive after 1 year [195]. Another study revealed that intestinal colonization in the general population persisted

for >8 months in 32.9 % [193]. However, a study amongst 633 Dutch travellers who acquired ESBL during travel, concluded that the median duration of colonisation after travel was 30 days, and only 14.3 % and 11.3 % remained colonized at 6 and 12 months after return, respectively [190]. A second large community survey showed that the average duration of carriage was 0.35 years (4.2 months) amongst 4177 Dutch community-dwelling subjects [192]. Spontaneous clearance of certain subclones appears to be more difficult, as colonization of *E. coli* subtype (ST) ST131 is associated with a longer duration of carriage in a long-term care facility residents, with a half-life of 13 months versus 2- to 3- months for other STs [196]. Of particular interest is the recognition of individuals with a higher risk of asymptomatic carriage and potential spread to the healthcare facility or community of MDROs. Nursing home residents as well as patients attending the hospital have multiple risk factors for colonization and infection with MDRO and *C. difficile* [178, 197-203]. They are thought to be a potential reservoir for spread and transmission in the hospital [80, 204, 205]. Frequent contact between residents due to communal living, high frequency of healthcare contact and presence of factors that facilitate MDRO spread such as incontinence present additional opportunities for transmission [206].

Resistance to polymixins and carbapenems

Infections with MDROs resulted in an increasing demand to use carbapenems. Subsequently, carbapenem resistance developed [207]. Carbapenemases can be produced by Enterobacterales and non-fermenters, and are classified into three classes according to Ambler classification; class A, B and D. Of the genes conferring carbapenem resistance, the carbapenemases pose the most threat, because of the possibility of horizontal gene transfer by plasmids or other mobile genetic elements. Carbapenemases are still sporadically observed in the Netherlands, both in the hospital [169] as well as in the community [194]. However, national surveillance detected a small cluster of eight closely related New Delhi metallo-beta-lactamase producing *K. pneumoniae* in patients without epidemiological link, indicating unnoticed spread [208]. Due to the rise of carbapenemase producing MDRO's, some of the older antibiotics such as polymyxins (colistin and polymyxin B) regained interest for patients' treatment. Colistin, also known as polymyxin E, is bactericidal and demonstrates activity against most Gram negative bacteria [209]. However, its nephrotoxicity

and neurotoxicity has prevented the use of colistin in regular patient treatment [210]. Therefore, colistin usage was mainly limited to veterinary medicine for treatment of gastrointestinal infections in food producing animals [211]. In the Netherlands, polymyxin B is frequently used for selective gut decontamination in Intensive Care Units and stem cell transplantation patients [212, 213]. The polymyxins are cationic peptides with fatty acid tails. Electrostatic interaction occurs between the positively charged groups on the polymyxin and the negatively charged groups of the lipid A component of the lipopolysaccharide (LPS). The fatty acids interact with the lipid components of the outer membrane. These interactions result in a change in the permeability of the outer membrane, and the polymyxins gain access to the cytoplasmic membrane. The breach in the permeability barriers, result in leakage of intracellular contents and subsequently cell death [209]. Some important Gram-negative bacteria, such as *Serratia marcescens*, *Burkholderia* and *Proteus* species, are intrinsically resistant to the action of polymyxins. Colistin resistance in Gram-negative bacteria is primarily due to post-translational modification of the lipopolysaccharide (LPS) molecules [214]. Substituents (such as 4-amino-$_{L}$-arabinose, phosphoethanolamine and/or galactosamine) which are positively charged reduce the negative charge of the outer membrane, resulting in less binding between the bacterial LPS and the colistin [214]. While the genes necessary for most of these additions are chromosomally encoded, the identification of a plasmid harbouring a novel colistin resistance gene, *mcr-1* in November 2015, is of concern as it threatens to increase the rate of colistin resistance [215]. Since the discovery of the *mcr-1* gene, ten *mcr* genes types (*mcr-1* to *mcr-10*) have been detected in Enterobacterales isolates of human, animal and environmental origin with worldwide distriution [216, 217]. The emergence of colistin resistance is currently analysed in a large European survey coordinated by ECDC and preliminary data indicate that its prevalence in the Netherlands amongst clinical isolates of *E. coli* and *K. pneumoniae* is higher than expected (March 2020, source RIVM).

Multidrug resistant organisms; rationale for microbiota modifying therapies

Most infections with ESBL producing Enterobacterales have high morbidity and mortality and are preceded by intestinal colonization [171, 177, 178]. Prevention and eradication of ESBL producing Enterobacterales from the intestinal tract is therefore of

interest. Since spontaneous decolonization occurs infrequently, innovative strategies for decolonization of MDR bacteria are needed. A published guidance document by the ESCMID could not recommend an antimicrobial intervention strategy for decolonization **[218]**. However, Millan et al., observed that FMT for treatment of patients with multiple recurrent CDI decreased the number and diversity of antimicrobial resistance genes **[219]**. This observation was followed by various case-reports of patients colonized with ESBL producing Enterobacterales who were successfully decolonized by FMT **[156-159, 220-225]**. However, only one RCT was performed which assessed decolonization of MDRO Enterobacterales by treatment of oral non-absorbable antibiotics or by FMT. Unfortunately, no statistically significant advantage of FMT was found, though the trial suffered from inclusion of insufficient number of patients **[160, 226]**.

Outline of this thesis

This thesis, entitled "Exploring the role of the microbiota in defence against *Clostridium difficile* and multidrug resistant Gram negatives", reports on the microbiological, epidemiological and clinical aspects of *Clostridioides difficile* and multidrug resistant organisms (MDROs). Part I of this thesis focusses on the epidemiology and diagnostic practices of asymptomatically colonized individuals, whereas part II focusses on eradication and/or treatment of these micro-organisms by restoring a healthy microbiota with "Faecal Microbiota Transplantation".

Chapter 1 is a general introduction on the gut microbiota in relation to colonization and infection with *C. difficile* and MDRO, and Fecal Microbiota Transplantation.

Part I: New insights in the epidemiology of *Clostridioides difficile* and multidrug resistant organisms.

Chapter 2 evaluates the performance of several diagnostic *C. difficile* tests compared to the gold standard toxigenic culture of asymptomatically colonized patients at admission to three large hospitals in the Netherlands.

Chapter 3 reports on the prevalence of plasmid mediated colistin resistance genes; *mcr-1,2* [215, 227], in faecal samples of patients attending a tertiary care hospital in the Netherlands. Furthermore, it describes the genetic mechanism of phenotypically colistin susceptible *mcr-1* containing *E. coli*.

Chapter 4 determines the prevalence, risk factors and transmission within the nursing home of *C. difficile* and MDRO in asymptomatic nursing home residents in a high (Ireland) and low (the Netherlands) endemic country. Transmission of MDROs was studied with whole genome sequence analysis.

Part II: The initiation of the Netherlands Donor Feces Bank to facilitate quality assured faecal microbiota transplantation

Chapter 5 describes the establishment of the Netherlands Donor Feces Bank (NDFB), a national operating non-profit stool bank facilitating FMT in the Netherlands. It addresses the current practice of donor recruitment and screening, preparation of the faecal suspension, logistics and transport of the faecal microbiota suspension to treating physicians in the local hospitals, and the follow-up of the outcome and safety of FMT in patients treated with FMT suspensions provided by the NDFB.

In **Chapter 6** the four years results of extensive donor screening and the outcome of FMT with suspensions provided by the NDFB are reported. In addition, the additional benefit of expert consultation, as provided by the working group of the NDFB is described. An attempt was made to understand the failures (post-FMT recurrence), and to identify donor and faeces suspension specific factors for optimal rCDI treatment.

Chapter 7 is an analysis of the effect of transmission of *Blastocystis* species from donors to patients by FMT, using a combination of PCR and subtyping techniques.

Chapter 8 describes an attempt of MDRO decolonisation from the intestinal tract with FMT in a patient suffering from recurrent urinary tract infections with a VIM-positive *Pseudomonas aeruginosa*. Microbiota analysis using 16S analysis was performed on both donor and patient stool before and after FMT.

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Part I

New insights in the epidemiology of *Clostridioides difficile* and multidrug resistant organisms

Chapter 2

Detection of *Clostridium difficile* in feces of asymptomatic patients admitted to the hospital

Diagnostics in asymptomatic *C. difficile* carriers

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Chapter 2. Detection of *Clostridium difficile* in feces of asymptomatic patients admitted to the hospital

Abstract

Recent evidence shows that patients asymptomatically colonized with *Clostridium difficile* may contribute to the transmission of *C. difficile* in healthcare facilities. Additionally, these patients may have a higher risk of developing *C. difficile* infection. The aim of this study was to compare a commercially available PCR directed to both toxin A as B (*artus C. difficile* QS-RGQ Kit CE, QIAGEN), an Enzyme Linked Fluorescent Assay to Glutamate Dehydrogenase (GDH ELFA, VIDAS, bioMérieux), and an in-house developed PCR to *TcdB*, with (toxigenic) culture of *C. difficile* as gold standard to detect asymptomatic colonization. Test performances were evaluated in a collection of 765 stool samples obtained from asymptomatic patients at admission to the hospital. The *C. difficile* prevalence in this collection was 5.1 %, 3.1 % contained a toxigenic *C. difficile*. Compared to *C. difficile* culture, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of *C. difficile* GDH ELFA were 87.2 %, 91.2 %, 34.7 % and 99.3 %. Compared with results of toxigenic culture, the sensitivity, specificity, PPV and NPV of the commercially available PCR and the in-house PCR were 95.8 %, 93.4 %, 31.9 %, 99.9 %, and 87.5 %, 98.8 %, 70 % and 99.6 %, respectively. We conclude that in a low prevalence setting of asymptomatically colonized patients, both GDH ELFA and a Nucleic Acid Amplification Test can be applied as a first screening test as they both display a high NPV. However, the low PPV of the tests hinders the use of these assays as stand-alone tests.

Introduction

Clostridium difficile infection (CDI) is a leading cause of hospital-acquired diarrhea. The transmission of spores from symptomatic patients can spread *C. difficile* within healthcare facilities with subsequent development of more symptomatic patients, and eventually clusters and outbreaks. However, recent data suggests that patients asymptomatically colonized with *C. difficile* also contribute to spread of *C. difficile* spores to the environment and to other patients [1-3]. Asymptomatic carriers shed spores into the environment to a lesser extent than CDI patients [3, 4], but by outnumbering the CDI patients they can still play an important role in the transmission of the disease. This hypothesis has recently been supported in a Canadian study, where isolation of *C. difficile* colonized patients significantly reduced the incidence of hospital-acquired CDI [5]. A second new insight in the significance of asymptomatic colonization is that it may increase the risk of subsequent clinical disease in some colonized patients [6-10]. Progression from colonization to CDI can be provoked by alterations of the microbiota and a subsequent decrease of secondary bile acids, which normally inhibit spore germination [11-13]. But other factors like pre-existing antitoxin antibodies may also play a role, in protection from progression to CDI, although their exact role needs to be clarified.

Thus, recognition of asymptomatically colonized patients may be clinically relevant to reduce nosocomial transmission and for protection from progression to symptomatic disease. Asymptomatic colonization of *C. difficile* varies widely between various patient populations studied. Approximately five to 15% of newly hospital admitted patients carry *C. difficile* in their feces [4, 5, 14-17]. Carriage rates of residents in long-term care facilities varies from 4-51%, but in general tends to be higher than in hospitalized patients [3, 14, 18]. Asymptomatic colonization of *C. difficile* in the pediatric populations is very high, approximately 37% of children are asymptomatic carriers in their first year of life, decreasing to 15% for children between one and eight years of age [19].

A recently published European guidance document advises a two-stage algorithm to diagnose CDI using a toxin Nucleic Acid Amplification Test (NAAT) or GDH Enzyme Immuno Assay (EIA) as sensitive screening assay in combination with tests to detect

the presence of free toxins in stools as a marker of disease activity [20]. Samples without free toxins detected will largely represent asymptomatic carriers. However, this guideline addresses diagnostics of CDI in diarrheal patients and reviewed the literature of symptomatic patients with CDI. The optimal diagnostic test to detect *C. difficile* in asymptomatically colonized patients with normally formed stool is unknown. Therefore, the aim of this study was to compare the performances of a commercially available glutamate dehydrogenase (GDH) EIA with a primary gold standard, a conventional culture of *C. difficile* in asymptomatically colonized patients at admission to three large hospitals in the Netherlands. Moreover a commercially available PCR for *TcdA* and *TcdB*, and in-house developed PCR for detection of *TcdB*, was compared with a secondary gold standard; toxigenic *C. difficile* culture (TC).

Material and methods

Study design

A multi-center study was performed on feces samples obtained between November 2014 and December 2015 in the Leiden University Medical Center, Leiden (623 samples), Amphia hospital, Breda (72 samples), and Erasmus Medical Center, Rotterdam (70 samples) in the Netherlands. The study was designed to determine risk factors for asymptomatic *C. difficile* colonization at admission to the hospital [ZonMW 50-52200-98-035]. The institutional review board judged that ethical approval was not required. Feces samples were obtained from patients on admittance to internal medicine and surgical wards, and from patients attending the kidney transplant outpatient clinic. If a patient was admitted twice in the study period, the patient remained eligible for this study.

Culture and characterization of *C. difficile*

The samples were processed for *C. difficile* culture and TC within 72 hours of arrival at the laboratory and were subsequently stored at -20°C, without addition of glycerol. Feces was inoculated on *C. difficile* selective agar (CLO-medium; bioMérieux, Marcy l'Etoile, France) and CNA- (colistin and naladixic acid containing agar, bioMérieux) medium with and without ethanol shock pretreatment [21]. The media were incubated for five days in

an anaerobic atmosphere at ± 35°C. Gray-brown colonies with the characteristic horse manure odor were further tested by an in-house GDH PCR [22]. *C. difficile* isolates were tested for the presence of toxin genes by PCR for toxin A (*TcdA*), toxin B (*TcdB*) and binary toxin (*CdtA*, *CdtB*) [22]. Capillary gel-based electrophoresis PCR ribotyping was performed to characterize the isolates [23].

Diagnostic *C. difficile* tests

After thawing the stored feces samples, the GDH EIA and both NAATs were performed in bulk testing. The targets of the applied detection assays are depicted in Table 1. The GDH EIA was performed on an enzyme-linked fluorescent immunoassay (ELFA) platform (VIDAS; bioMérieux, Marcy-l'Etiole, France) as previously described [24]. A relative fluorescent value of ≥ 0.1 was regarded positive. Both GDH ELFA and *artus* PCR were performed according to manufacturer's instructions. As both assays are not registered for use in formed stools of asymptomatic patients, instructions were modified for off-label use, in consultation with the manufacturer. For both tests approximately the size of half a pea of feces (approximately 0.3 to 0.4 gram), instead of 200 µl of liquid feces, as this is routine practice in our laboratory for isolation of DNA of formed feces. For the *artus* PCR (*artus C. difficile* QS-RGQ Kit CE, QIAGEN, the Netherlands) feces was transferred into a test tube with 1500 µl tissue lysis buffer (ATL), vortexed and centrifuged for a short period. The tubes were then inserted into the QiaSymphony supplied with the *artus C. difficile* AS software, which regulates DNA isolation and preparation of PCR mix. The PCR mix was manually transferred to the Rotor Gene Q MDx. Samples with invalid *artus* PCR results were retested until the result was valid, with a maximum of three testing rounds. For the in-house PCR, DNA extraction was performed using the MagnaPure96 system (Roche Diagnostics, Almere, the Netherlands). In short, approximately half a pea size of feces was resuspended in 1 ml S.T.A.R. buffer (Roche, Diagnostics), supplemented with Precellys beads (Bertin Technology, France), mixed thoroughly by shaking on a Vibrax shaker (5 min, 2200 rpm) and centrifuged for 1 min at 14000 rpm. Of the supernatant, 200 µl was used for nucleic acid (NA) extraction using the MP96 DNA and Viral NA Small volume kit yielding a final eluate of 100 µl. The in-house developed real-time PCR for the specific detection of the *TcdB* gene was tested in a multiplex assay with Phocine herpes virus as internal control as described previously [25]. Samples with

a quantification cycle (*Cq*) value higher than 40 were considered negative. In addition, samples with an internal control *Cq* value that deviated more than 3.3 *Cq* values compared to the internal control *Cq* value of the negative control were considered inhibited. Due to a change in workflow of adding BSA to all our in-house PCRs with feces as sample material to decrease the inhibition rate, the last 142 samples were tested with addition of 5 mg/mL of the PCR enhancer bovine serum albumin (BSA) [26].

Table 1. *C. difficile* detection assays included in this this study

Assay type	Assay	Target(s)	Supplier
Anaerobic culture	*C. difficile* culture	Identification by PCR with GDH as target	In-house [22]
Toxigenic culture	*C. difficile* culture and PCR for toxin genes	Multiplex PCR with *TcdA*, *TcdB*, *cdtA* and *cdtB* (binary toxin)	In-house [22]
Automated immunoassay	Vidas GDH	GDH	Biomerieux, France
Nucleic acid amplification test	Artus *C. difficile* QS-RGQ Kit CE	*TcdA* and *TcdB*	QIAGEN, Germany
Nucleic acid amplification test	In-house *C. difficile* PCR	*TcdB*	In-house [25]

GDH: Glutamate Dehydrogenase, tcdA: toxin A, tcdB: toxin B, cdtA and cdtB: binary toxin

Discrepancy analysis

Samples with discordant results were retested, except for positive results of the GDH ELFA because of an expected low specificity. An enriched TC was performed when three diagnostic tests were positive and the TC was negative. For enriched TC, half a pea size of feces was suspended in a cycloserin-cefoxitin-mannitol broth with taurocholate, lysozyme and cysteine (CCMB-TAL, Anaerobe Systems, Morgan Hill, CA). The enrichment broth was subcultured on CLO and CNA agar as described above, on day two and five.

Statistical analysis

A GDH positive result was considered true positive or true negative, if the stool culture was positive or negative for *C. difficile*, irrespective of its toxin production. For both PCRs a positive result was considered true positive or true negative if the stool culture was positive or negative for toxigenic *C. difficile*. False positive and false negative test results were defined as discrepant results compared to the gold standard. Sensitivity and specificity of the tests were determined by the proportion positive, or negative respectively, correctly identified. The difference in both sensitivity and specificity between the toxin PCRs was determined using McNemar's test for paired proportions. The sensitivity and specificity data were used to calculate the positive predictive value (PPV) and negative predictive value (NPV). *Cq* values of false positive results were compared with *Cq* values of true positive results using an independent student t-test. ROC curves were constructed for all index tests. Analyses were performed using SPSS 23.0 and STATA version 12.1 statistical software.

Results

In total, 765 feces samples of 581 unique patients were included in the evaluation, of which 39 (5.1%, 95% CI: 3.8-6.9) were positive for the presence of *C. difficile* by culture; 24 (3.1%, 95% CI: 2.1-4.6) contained toxigenic *C. difficile*. All 765 samples were tested by toxigenic culture, GDH ELFA and in-house PCR, but due to insufficient sample volume of one sample, 764 samples were tested with the *artus* PCR. Sensitivity, specificity, PPV and NPV data of the various tests are depicted in Table 2. The *artus* PCR had the highest sensitivity of 95.8%. The mean *Cq* value in true positive samples was 27.5 for *TcdA* and 28.4 for *TcdB*. The in-house PCR showed an sensitivity of 87.5%, with a mean *TcdB Cq* value of 29.3 in true positive samples. The difference in sensitivity between *artus* PCR and the in-house PCR was not significant (p=0.5). The GDH ELFA had a sensitivity of 87.2%. The mean relative fluorescent unit (RFU) of the GDH ELFA in true positives was 11.7 (SD 8.11). Specificities were 98.8%, 93.4% and 91.2% for the in-house PCR, *artus* PCR and GDH test, respectively. The specificity of the in-house PCR was significantly higher than the *artus* PCR (p<0.000001). The NPV was in general very high and ranged from 99.3% to 99.9% for all assays. The PPV, on the other hand, was only 31.9% for the

artus PCR and 34.7 % for the GDH ELFA. In comparison to these former two tests, the in-house PCR had a higher PPV of 70.0 % (Table 2). Receiver operating characteristics (ROC) curves were made for the performances of the individual tests. For GDH ELFA, *artus* PCR and in-house PCR, the diagnostic accuracy as given by the area under the curve was 0.8918, 0.9467 and 0.9314, respectively *(supplemented Figure 1)*.

Table 2. Comparison of various *C. difficile* detection assays in comparison with culture of toxigenic and non-toxigenic *C. difficile* as gold standards. The sensitivity and specificity are given as percentages, and the 95 % confidence interval (95 % CI) values are shown in parentheses.

	(Toxigenic) Culture [1]					
Assay	**Pos**	**Neg**	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**	**PPV (%)**	**NPV (%)**
GDH positive	34	642	87.2 (72.6-95.7)	91.2 (88.9-93.1)	34.7	99.3
GDH negative	5	662				
Artus positive	23	49 [2]	95.8 (78.9-99.9)	93.4 (91.3-95.1)	31.9	99.9
Artus negative	1	691				
In-house positive	21	9 [2]	87.5 (67.6-97.3)	98.8 (97.7-99.4)	70	99.6
In-house negative	3	732				

1 GDH ELFA was compared with *C. difficile* culture, *Artus* and in-house PCR were compared with toxigenic culture.
2 Four of false negative samples were positive in all tests (GDH, *Artus*, in-house PCR)

Of 623 samples tested with the in-house PCR without the addition of BSA, 61 (9.8 %) showed inhibition which disappeared at a 1:10 dilution of the sample. Of 142 samples tested by the in-house PCR with addition of BSA, no inhibition was observed. Of 764 samples tested by *artus*, 40 (5.2 %) showed inhibition which disappeared with repeated testing. Additionally, 26 (3.4 %) invalid *artus* results were obtained due to *TcdA Cq* value above accepted range (n = 4), a *TcdB Cq* value above accepted range (n = 16), *TcdA* uncertain (n = 3) or *TcdB* uncertain (n = 3).

A discrepancy analysis was performed on discordant results and is displayed in Table 3. Four of 741 TC negative samples tested positive with all three assays (GDH ELFA, in-house PCR and *artus* PCR), suggesting a false negative result of the TC. One of these 4 feces samples was positive for culture of toxigenic *C. difficile* using the enriched TC method, suggesting that a very low number of *C. difficile* was present. Two

Table 3. Results of first and second tests

After resolving of the four TC negative/other tests positive samples, results were as follows: two false positive in-house PCR results retested positive (Cq values 28 and 31.6 respectively), while three false positive in-house PCR results could not be confirmed with retesting. All 45 false positive artus samples retested negative. All remaining 60 GDH false positive samples were not retested. Two out of three false-negative in-house PCR results retested positive. One in-house PCR and artus false negative sample remained negative upon retesting by both PCRs, while both in-house and artus PCR on the cultured strain were positive. Two GDH ELFA negative sample retested positive, while two remained negative.

No of specimens	First tests					Second test(s)			
	GDH	in-house	artus	culture	TC	GDH	in-house	artus	enriched TC
617	–	–	–	–	–	ND	ND	ND	ND
56	+	–	–	–	–	ND	ND	ND	ND
40	–	–	+	–	–	ND	ND	–	ND
19	+	+	+	+	+	ND	ND	ND	ND
12	+	–	–	+	–	ND	ND	ND	ND
4	+	–	+	–	–	ND	ND	–	ND
2	–	++	–	–	–	ND	–	ND	ND
2	–	+	–	–	–	ND	+	ND	ND
2	–	–	–	+	–	+	ND	ND	ND
1	–	+	+	+	+	+	ND	ND	ND
1	–	+	+	+	+	–	ND	ND	ND
1	–	–	+	+	+	–	+	ND	ND
1	+	–	+	+	+	ND	+	ND	ND
2	+	+	+	–	–	ND	ND	ND	ND
1	+	+	+	–	–	ND	ND	ND	+
1	+	+	+	–	–	ND	+	+	–
1	+	+	+	+	–	ND	–	–	–
1	+	–	–	+	+	ND	–*	–*	+
1	–	–	ND	–	–	ND	ND	ND	ND

The green color indicates a false (positive or negative) result

ND: not detected

* In house and artus PCR positive on strain, not on feces

other TC negative results could be explained by vancomycin treatment at time of feces sampling, which inhibits the growth of *C. difficile*. No clear explanation was found for the remaining false negative TC feces sample. All 45 false positive *artus* samples tested negative with retesting. The *artus* PCR flags a result as real positive when one or both of the toxin genes are below a certain *Cq* value. Of these 45 TC negative but positive flagged *artus* results 21 were positive only for *TcdA*, and seven only for *TcdB*, whereas 17 were both *TcdA* and *TcdB* positive. The mean *Cq* values of the false positives were higher (*TcdA* 33.1, *TcdB* 33.4) than the *Cq* values of *artus* true positives (*TcdA* 27.8, *TcdB* 28.4). No discrepancy analysis was performed on the remaining 60 GDH false positive samples (Table 2; 60 minus four as mentioned above) due to the expected low specificity. One feces sample tested negative by in-house PCR as well as the artus PCR and remained negative with retesting. However, the toxigenic cultured strain was positive tested by both PCRs, suggesting that a very low number of *C. difficile* was present in the feces. After the discrepancy analysis the sensitivity, specificity, PPV and NPV were 96.6 %, 100 %, 100 %, 99.9 % and 96.9 %, 99.7 %, 93.3 % and 99.7 % of *artus* and in-house PCR respectively.

The distribution of PCR ribotypes isolated from asymptomatic patients in this cohort is displayed in *Figure 1*. Five strains could not be ribotyped since the profiles of the corresponding strains were not present in the National Reference Laboratory of the Netherlands.

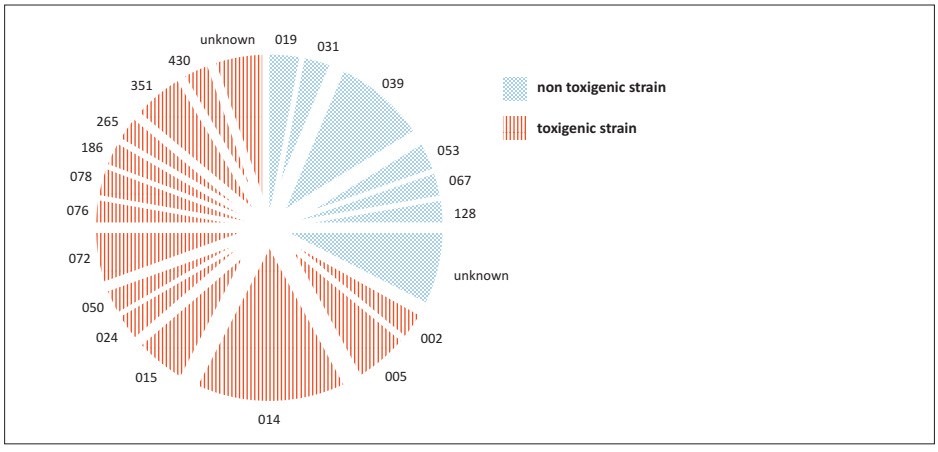


Figure 1. Distribution of *C. difficile* ribotypes isolated from asymptomatic patients displayed in a pie chart. Indicated in red (arched) are the toxigenic strains, in blue (dotted) the non-toxigenic strains. The numbers indicate the corresponding ribotype number.

Discussion

The aim of this study was to compare two molecular assays (*artus C. difficile* PCR, in-house *TcdB* PCR) and *C. difficile* GDH ELFA with (toxigenic) culture of *C. difficile* as gold standards to detect asymptomatic colonization.

In this study, 5.1% of the patients attending a tertiary care hospital was positive with *C. difficile* and 3.1% contained a toxigenic *C. difficile*. Other studies testing feces samples for the presence of asymptomatic colonization of *C. difficile* at admission (or collected feces within 72 hours after attending the hospital), reported a higher prevalence of 7.5%-15.7% toxigenic *C. difficile* **[1, 2, 10, 27-30]**. The lower prevalence rate in our study is probably related to the overall low prevalence of *C. difficile* and CDI in the Netherlands. A recently completed cross-sectional study among 2,494 healthy adults in the Netherlands revealed a prevalence of toxigenic *C. difficile* of 1.2% in the community (submitted).

The sensitivity and specificity of the automated VIDAS GDH ELFA in comparison to *C. difficile* culture were 87.2% and 91.2%, respectively. Davies et al, studied the performance of the same GDH ELFA in diarrheal samples submitted for *C. difficile* testing, and reported a higher sensitivity of 95.8% and a similar specificity of 91% **[31]**. The lower sensitivity found in our study could be due to presence of lower numbers of *C. difficile* in feces samples of asymptomatically colonized patients than in patients with CDI **[18]**. However, we don't exclude the possibility that the percentages change when larger number of feces samples are tested. An alternative explanation for the lower sensitivity rates in our study are the formed feces samples that we included, instead of diarrheal samples.

The *artus* PCR and the in-house PCR were compared with TC and revealed sensitivities of 95.8% and 87.5% respectively, though this difference in sensitivity rate was not significant. In contrast, the *artus* PCR was statistically less specific in comparison with the in-house PCR (93.8% versus 98.8%). Since the *artus* PCR positive, TC negative samples could not be confirmed by retesting, the results indicate false positivity. This was supported by the considerably higher *Cq* values of *TcdA* and *TcdB* for the false positive test results than for true positives. A hypothetical algorithm

to enhance the specificity of *artus* is considering the *artus* PCR result only positive when *TcdA* as well as *TcdB* are positive. This, resulted in a specificity of 97%, and a subsequent PPV of 51%, while remaining the high sensitivity of 95.8%. However, in the rare event a patient is colonized with a toxin A negative, toxin B positive *C. difficile* strain [32, 33], the new strategy will not identify this strain. The test results of *artus* showed less inhibition rates than the in-house PCR, 8% versus 5.3%, however inhibition of the in-house PCR was overcome by adding the PCR enhancer BSA [26]. An additional 3.5% of *artus* tested samples gave invalid test results, largely because of *Cq* values above the accepted range. Because of invalid or inhibited results, 8.7% of the feces samples needed retesting by the *artus* PCR. The performance of the *artus* PCR in this study resembled the results in loose stool samples submitted for CDI testing as reported by Jazmati et al [34]. In a collection of 201 stools specimens all 28 positive TC samples were detected by the artus PCR (sensitivity 100%), but the specificity, similar to this study, was relatively low (89.5%) [34]. They stated that the lower specificity could largely be explained by a higher sensitivity of the *artus* PCR than TC. However, we did not share this observation. Our hypothesis is that the false positive results were based on DNA contamination, as none of the other diagnostic tests were positive in these samples. The contamination route can be explained by the manual handling of the tubes containing the isolated DNA and PCR mix to the Rotor Gene, which required placing caps on tiny tubes, arranged very close to each other. The sensitivity of both PCR's and specificity of the in-house PCR are in agreement with the NAAT test performance in symptomatic *C. difficile* patients, as mentioned in the recently published ESCMID guidance document with an overall sensitivity of 95% and specificity of 98% in comparison with TC [20]. A discrepancy analysis was performed, mainly to clarify why conflicting test results were obtained. The test characteristics that were calculated after resolving discrepant results could thereby be biased in favor of the index tests and should be considered with caution.

For CDI diagnosis, the use of a two-step algorithm is recommended [20]. After a first sensitive test which reliably classifies non-diseased patients, a more specific test is applied as a second test to discern true positives from false positives. For diagnosis of colonized patients, a similar approach could be used. All three assays that we analyzed in this study had high NPV and would therefore be useful as a first screening test. Thereafter, confirmation of positive samples by a specific test could be

recommended. This specific test could be a NAAT or toxigenic culture when GDH was used as a first screening test. A second algorithm could be screening by NAAT and confirmation by TC.

The ribotypes of asymptomatic carriers found in this study, do not differ from the ribotypes found among CDI patients [35]. This supports the hypothesis that asymptomatic carriers and CDI patients share a common source, or transfer *C. difficile* to each other irrespective of the PCR ribotype. Furthermore only one of 24 *C. difficile* strains belonged to the epidemic ribotype 078 and no 027 strains were detected. Other studies confirm this finding [36-38], supporting the hypothesis that epidemic strains seldom lead to asymptomatic colonization.

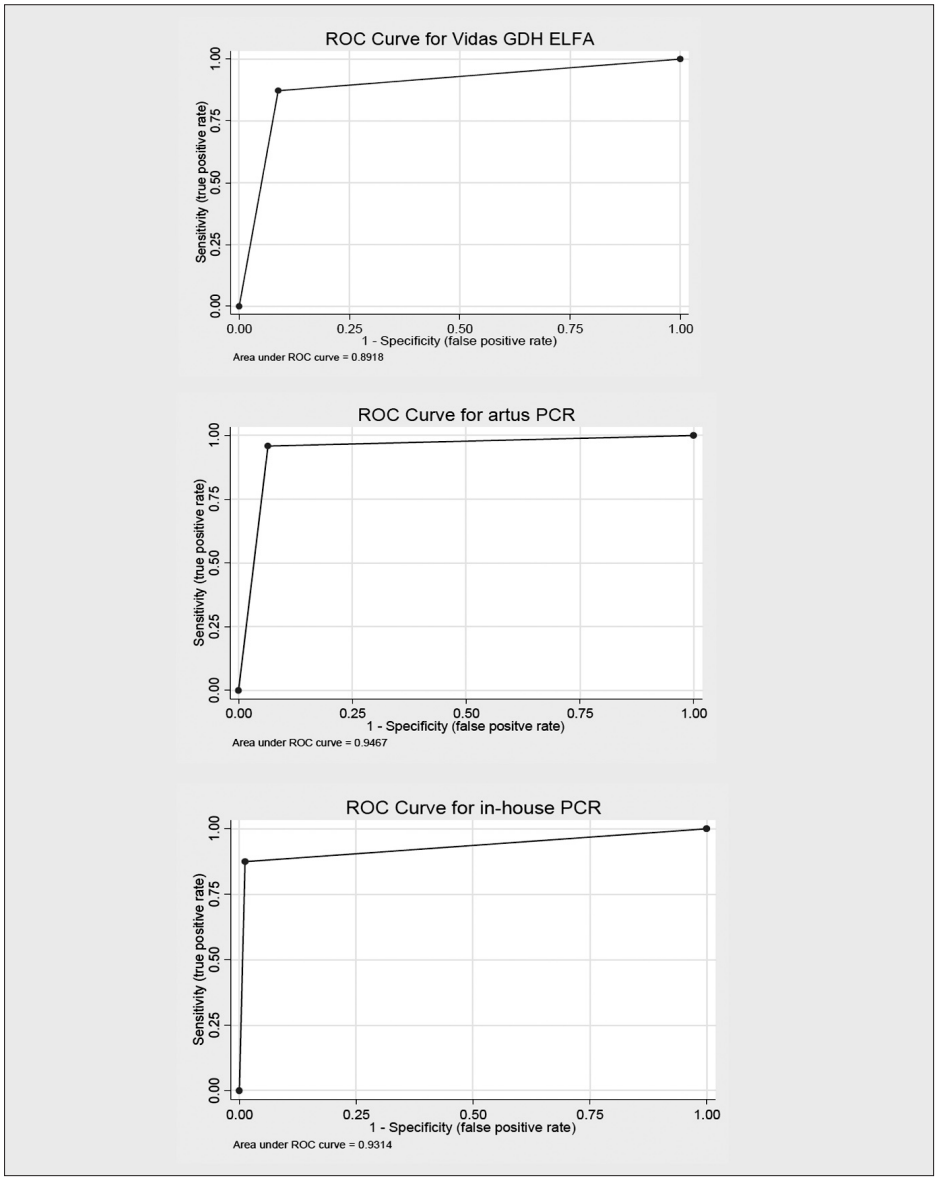
Many studies report on the performance of *C. difficile* diagnostic assays in patients with presumed CDI, but only a few report on the application of diagnostic tests in patients with asymptomatic *C. difficile* colonization [1, 2, 15, 27, 28]. The studies in asymptomatically colonized patients vary greatly in patient inclusion criteria, tested material and applied diagnostic and gold standard tests. For instance, a great number of the studies only test rectum swabs, or use a combination of stool samples and rectum swabs [4, 8, 18, 27, 28, 36, 39]. Guererro et al. showed that asymptomatic carriers have lower numbers of *C. difficile* in their rectal swab than CDI patients, indicating that stool samples should be preferred [4]. Furthermore, a mix of diagnostic screening tests have been applied to detect *C. difficile*, frequently subdivided into assays to recognize toxigenic or non-toxigenic strains [20]. However, a comparison of various diagnostic tests with a reference method to detect asymptomatic colonization of *C. difficile* has not been studied before.

Our study has a few limitations. An important limitation is the low prevalence rate of asymptomatic *C. difficile* colonization, which resulted in a low PPV of 31.9 to 70 % for the different tests. However, this prevalence rate provides the most precise information on the performance of the test in our patient population. All tests would have better PPVs in a population with higher prevalence rates of *C. difficile* colonization, or when a selection of samples is tested when a predictive model for *C. difficile* colonization becomes available. A second limitation may be the freeze-thaw step which presumably can lower the sensitivity, though we performed all tests immediately after thawing,

except for the discrepancy analysis. In addition, no published reports indicate that free-thawing affect the performances of diagnostic PCRs for bacterial pathogens.

In conclusion, this study is the first which evaluates the use of three different assays for detection of asymptomatic *C. difficile* colonization in stool samples and compares it to their gold standards. In our low endemic setting of asymptomatically colonized patients all three assays (i.e. GDH ELFA, *artus* PCR and in-house PCR) can be applied as a first screening test as they display a very high NPV. The positive predictive values of these tests were suboptimal and therefore these assays are not suitable as stand-alone tests in a low prevalence setting.

Supplementary information



Supplementary Figure 1. Receiver operating characteristics (ROC) curves were for the performances of the individual tests.

For GDH ELFA (top), artus PCR (middle) and in-house PCR (bottom), the diagnostic accuracy as given by the area under the curve was 0.8918, 0.9467 and 0.9314, respectively.

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Potential conflicts of interest

None to declare.

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Chapter

3

Prevalence of colistin resistence gene (*mcr*-1) containing Enterobacteriaceae in feces of patients attending a tertiary care hospital

and detection of a *mcr*-1 containing, colistin susceptible *E. coli*

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Chapter 3. Prevalence of colistin resistence gene (*mcr-1) containing Enterobacteriaceae in feces of patients attending a tertiary care hospital and detection of a mcr-1* containing, colistin susceptible *E. coli*

Abstract

The emergence of the plasmid-mediated *mcr* colistin resistance gene in the community poses a potential threat for treatment of patients, especially when hospitalized. The aim of this study was to determine the prevalence of all currently known *mcr* mediated colistin resistance gene in fecal samples of patients attending a tertiary care hospital. From November 2014 until July 2015, fecal samples of patients attending the Leiden University Medical Center were collected and screened for presence of *mcr* using real-time PCR. Two of 576 patients were positive for *mcr-1*, resulting in a prevalence of 0.35 %, whereas no *mcr-2* was found. One of these samples was culture negative, the second sample contained a *bla*CMY-2 and *mcr-1* containing *E. coli*. This strain belonged to Sequence Type 359 and serotype O177:H21. The *mcr-1* containing *E. coli* was phenotypically susceptible to colistin with a MIC of ≤ 0.25mg/l, due to a 1329bp transposon IS10R inserted into the *mcr-1* gene as identified by WGS. This prevalence study shows that *mcr-1* is present in low levels patients out of the community attending a hospital. Furthermore the study underlines the importance of phenotypical confirmation of molecular detection of a *mcr-1* gene.

Introduction

Colistin, also known as polymyxin E, is highly active against most Gram-negative bacteria [1]. However, its nephrotoxicity and neurotoxicity has prevented the use of colistin in regular patient treatment [2]. Therefore, colistin usage was mainly limited to veterinary medicine for treatment of gastrointestinal infections in food producing animals [3]. In the Netherlands, colistin is frequently used for selective gut decontamination in Intensive Care Unit (ICU) and stem cell transplantation patients [4, 5]. Colistin regained new worldwide interest after the emergence of multidrug resistant (MDR) *Enterobacteriaceae* and is nowadays used as a last resort antibiotic for infections caused by MDR *Enterobacteriaceae*. The recent finding of a plasmid harbouring a novel colistin resistance gene, *mcr-1* and *mcr-2*, is therefore of concern [6, 7].

The *mcr-1* colistin resistance gene is predominantly found in *Enterobacteriaceae*, and results in a moderate level of resistance, with MIC values varying from 4 to 16 mg/l [8, 9]. The prevalence of *mcr-1* varies considerably and ranges from 0.02 % to 20.6 % in livestock, 1.3 % to 19 % in retail meat and 0.08 % to 2 % in hospitalized patients [10-14]. The worldwide distribution of the *mcr-1* gene and a relatively high prevalence of *mcr-1* mediated colistin resistance in livestock and retail meat suggests food animals as reservoir for transmission to humans [8]. Until now, almost exclusively Extended Spectrum Beta-Lactamase (ESBL) producing or colistin resistant isolates have been screened for the presence of *mcr-1*. A number of reports of *mcr-1* in the United States of clinical and ESBL-negative strains indicate that the true extent of *mcr-1* prevalence amongst unselected Gram-negatives may be highly underestimated [15, 16]. The *mcr-2* colistin resistance gene had 76.7 % nucleotide identity to *mcr-1* and had so far only been found in colistin-resistant *E. coli* isolates identified from porcine and bovine [7].

Studies about human fecal carriership of *mcr-1* in the community are limited, and so far only been described in China; in healthy volunteers (prevalence of 19 of 2923 = 0.65 %), in a public bacterial metagenome dataset before 2010 (prevalence of 3/1267 = 0.24 %) [17, 18], and in Dutch travellers returning from Asia, South America or Africa (prevalence of 0.95 % - 4.9 %) [19, 20]. Recently, no *mcr* genes could be detected in the stool of 1091 healthy Swiss individuals [21]. Epidemiological data on the prevalence of *mcr-1* in the community attending a hospital are lacking and the risk of

colonized patients to spread *mcr-1* positive bacteria is unknown. Therefore, the aim of this study was to determine the prevalence of *mcr* mediated colistin resistance gene in fecal samples of patients attending a tertiary care hospital.

Material and methods

Patients and specimens

Between November 2014 and July 2015, fecal samples were obtained from patients on admittance to internal medicine and surgical wards, and from patients attending the kidney transplant outpatient clinic of the Leiden University Medical Center (LUMC) in the Netherlands. These wards were selected for their relative high patient turn-over, enabling more rapid inclusion of sufficient patients attending our hospital. The fecal samples were originally used for a study to define the role of *Clostridium difficile* in asymptomatic colonised patients at admittance to the hospital. The samples were processed within 72 hours of arrival at the laboratory and were subsequently stored at -20°C, without addition of glycerol. These samples obtained for *C. difficile* screening were also used for screening of the *mcr* gene. The medical ethical committee "Medisch Ethische Toetsings Commissie" of the LUMC waived the need for consent for the additional analysis on these fecal samples.

DNA extraction and real-time PCR

After thawing the stored fecal samples, DNA extraction was performed using the MagnaPure96 system (Roche Diagnostics, Almere, Netherlands). In short, approximately 0.3 to 0.4 gram (half a pea) feces was resuspended in 1mL S.T.A.R. buffer (Roche Diagnostics, Almere, The Netherlands), supplemented with Precellys beads (Bertin Technology, France), mixed thoroughly by shaking on a Vibrax shaker (5 min, 2200 rpm) and centrifuged for 1min at 14000 rpm. Of the supernatant, 200µl was used for nucleic acid (NA) extraction using the MP96 system and Viral NA Small volume kit (Roche Diagnostics) yielding a final eluate of 100µl. To monitor the NA extraction process and the presence of potential PCR inhibitors in the eluate, an universal internal control Phocine Herpes Virus (PhHV) was used [22]. Initially real-time PCR for the specific detection of the *mcr-1* gene was tested in a multiplex assay with PhHV as

described previously [23]. After the report of Xavier *et al.*, describing *mcr-2*, a generic *mcr* real-time PCR assay for the detection of both *mcr-1* and *mcr-2* was developed and used to screen for the presence of additional *mcr-2* containing samples (Table 1) [7].

Table 1. Primers and probe used to screen for the presence of mcr-genes

Oligonucleotide	Sequence (5'-3')	PCR product
Mcr-generic fw	GCCAAATACCAAGAAAATG	
Mcr-generic probe	TATCACGCCACAAGATAC	98bp
Mcr-generic rev	TTATCCATCACGCCTTTT	

Culture and colistin susceptibility testing

To further characterize *mcr* containing isolates, *mcr* positive fecal samples were cultured on commercially available sheep blood-, CNA- (colistin and naladixic acid containing agar) and CLED- (cysteine lactose electrolyte deficient) medium (Bio-Mérieux, Marcy l'Etiole, France) both directly and after enrichment in a Tryptic Soy Broth with and without colistin (2 mg/l). All morphological different aerobic Gram-negative bacteria were identified by MALDI-TOF MS (Microflex, Bruker Daltonics, Bremen, Germany) and tested for the presence of *mcr-1* by real-time PCR as described earlier. All bacterial isolates were also tested for colistin resistance with VITEK2 (card N199, BioMérieux, Marcy l'Etiole, France) and Sensititre colistin microdilution assay (Sensititre, TREK Diagnostic Systems, Inc., Cleveland, OK), using EUCAST breakpoints for *Enterobacteriaceae*, which interprets a MIC of ≤2 mg/l as susceptible and >2mg/l as resistant.

Whole Genome Sequence analysis

Whole Genome Sequence analysis of *mcr-1* containing isolates was performed to further characterize the *E. coli* strain including the plasmid carrying the *mcr-1* gene and other genes associated with antimicrobial resistance [6]. The genome sequence of the *mcr-1* containing isolate was determined using the Pacific Biosciences *RS*II system from DNA prepared by the Qiagen Genomic Tip 500/G kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. *De novo* assembly was performed using SMRT®Analysis v2.3.0 (PacBio's bioinformatics software suite) with expected genome

size of 5 Mbp and coverage of 30. The assembled sequence was analysed using Geneious software V8.0.5 (Biomatters, Auckland, New Zealand) and the online tools Resfinder, MLST, SeroTypeFinder and Plasmidfinder (http://genomicepidemiology.org/). The plasmid sequence was analysed in DNA plotter to generate a circular DNA map.

Results

Mcr prevalence and culture of *mcr* containing isolates

A total of 621 fecal samples of 576 unique patients were screened for presence of the *mcr* genes by real-time PCR. The median age of patients at submission of their stool was 62 years (range 18-93). Two samples of two different patients (0.35 %) were positive for *mcr-1* in real-time PCR with quantification cycle (Cq) values of 31 and 17, respectively (S1, Table). Additional testing with the *mcr*-generic real-time PCR assay confirmed this finding and did not find extra positive samples. A *mcr-1* containing *E. coli* isolate was cultured from the second fecal sample (Cq 17) only, in subcultures of the enrichment broth without colistin. Remarkably, despite the presence of *mcr-1* gene sequences, this *E. coli* isolate tested colistin susceptible (MIC <0.25 mg/l), which was confirmed in triplicate by both VITEK2 and the Sensititre assay. The antimicrobial susceptibility results and the corresponding genes coding for resistance are depicted in Table 2. Because of the decreased susceptibility to cephalosporins, the production of ESBL was tested phenotypically using the combination disk diffusion test, with a negative result. Subsequent testing for an AmpC β-lactamase gene by an in-house developed real-time PCR assay showed the presence of the *bla*CIT gene.

Table 2. Antibiotic phenotype with the corresponding molecular resistance of cultured *mcr-1* containing *E. coli*

The phenotype was tested with VITEK2 and a colistin microdilution assay, using EUCAST breakpoints. Molecular resistance determined with whole genome sequencing.

Antibiotic	MIC (mg/l)	Interpretation	Encoding resistance genes
Ampicillin	≥ 32	R	blaTEM-1B
Amoxicillin/ Clavulanic acid	≥ 32	R	blaTEM-1B
Cefuroxime	32	R	blaCMY-2
Cefotaxime	4	R	blaCMY-2
Cefoxitin	≥ 32	R	blaCMY-2
Ceftazidime	16	R	blaCMY-2
Cefepime	≤ 1	S	
Ciprofloxacin	≥ 4	R	
Colistin	≤ 0.25	S	*Mcr-1* inserted by IS10R transposon
Gentamicin	≤ 1	S	*aph(3')-Ic, strB, strA, aadA5*
Meropenem	≤ 0.25	S	
Nitrofurantoin	≥ 320	S	
Piperacillin/Tazobactam	≤ 4	S	
Tetracycline	128	R	*tetB*
Tobramycin	≤ 1	S	*aph(3')-Ic, strB, strA, aadA5*
Trimethoprim/Sulfamethoxazole	≥ 4	R	*sul1, sul2, dfraA17*

Whole Genome Sequence analysis

WGS analysis showed that the *mcr-1* gene found in the colistin susceptible *E. coli* isolate had a homology of 100 % with the first published *mcr-1* gene sequence [6]. However, the reading frame was disrupted by a 1329bp long IS10R transposon *(Figure 1)*. WGS analysis of the *E. coli* resulted into six contigs with a total length of ~5.5 Mbp (accession numbers: CP016546-CP016551, S2 Table). The largest contig was ~5.1 Mbp, covering the expected *E. coli* genome size, whereas analysis of the remaining five contigs (length between ~7.3 kb and ~126 kb) with PlasmidFinder 1.3 indicated the presence of plasmids IncX4 (~50 kb), IncI2 (~86 kb) , IncB/O/K/Z (~91 kb) and IncY (~126 kb). WGS analysis also revealed the presence of two identical IS10R containing *mcr-1* genes located on the same IncX4 plasmid. Multi Locus Sequence Typing (MLST) and serotype analysis showed that the *E. coli* belonged to Sequence Type (ST)

359 and serotype O177:H21. With ResFinder, the AmpC belonging to the CIT-group, as detected by the in-house AmpC real-time PCR, was confirmed to be present as *blaCMY-2*, located on the plasmid designated as IncB/O/K/Z. Additional genes associated with antimicrobial resistance detected in the sequence with their resulting antimicrobial phenotype are depicted in Table 2.

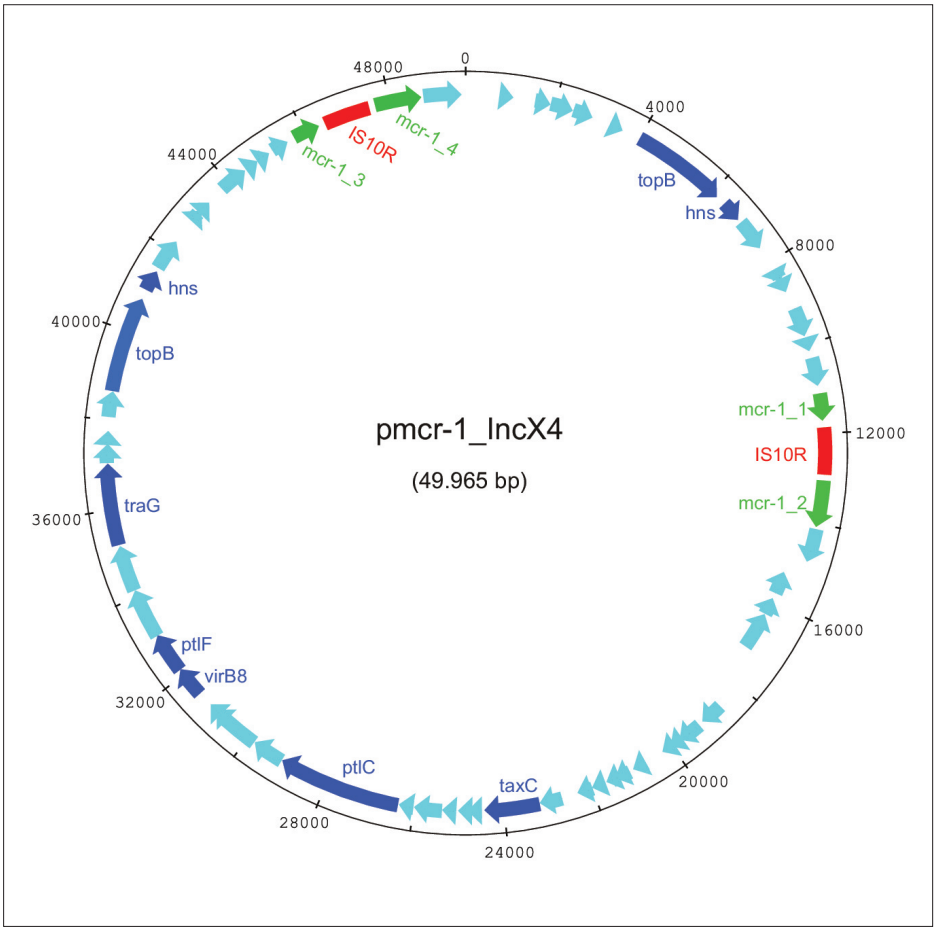

Figure 1. Circular presentation of the *mcr-1* containing IncX4 plasmid in the colistin susceptible *E. coli*

In green the *mcr-1* sequence. In red the IS10R insertion sequence, interrupting the *mcr-1* gene at position 572. Arrows indicate open reading frames (ORFs), dark blue ORFs with annotation, light blue ORFs without annotation (hypothetical protein). Numbers indicate nucleotide positions.

Patient characteristics

Both *mcr-1* positive patients were kidney transplant patients. The *mcr-1* positive stool sample from which no *mcr-1* containing isolate could be cultured belonged to a patient admitted to the acute care ward due to bacteremia with a colistin resistant *Salmonella enterica* serotype *Dublin* (MIC ≥4 mg/l). The *S. enterica* isolate tested negative with the specific *mcr-1* PCR. The feces with the colistin susceptible *mcr-1 E. coli* belonged to a patient attending the kidney transplant outpatient clinic. No epidemiologic link could be established between the two patients. The patients did not have a history of recent travelling and had not been treated with colistin recently. Also, none of the patients had developed an infection with a *mcr-1* containing isolate.

Discussion

To assess the risk of *mcr* introduction into our academic tertiary care hospital, the prevalence of *mcr* in fecal samples obtained from patients attending our hospital was investigated and found to be 0.35 % (n=2) of the 576 tested patients for *mcr-1*, whereas no *mcr-2* was found. This low prevalence is in accordance with earlier studies performed in asymptomatic carriers in the European community, ranging from 0 % to 0.92 % [19-21]. However, studies on *mcr-1* prevalence in asymptomatic carriers attending a hospital are lacking. Infections in hospitalized patients with *mcr-1* positive isolates have been reported in a number of countries, ranging from 0.24 % to 1.4 % depending on the used denominator [6, 10, 24]. In line with earlier studies, no *mcr-2* containing samples were detected in this study [21, 25].

One of the *mcr-1* positive fecal samples from the current study could not be confirmed by culture, most likely due to the fecal storage without glycerol at -20°C for one year which reduces the viability of Gram-negative bacteria. The fecal sample of the second patient contained a *mcr-1* positive *E. coli* with a colistin MIC of <0.25 mg/l. WGS analysis of the isolate revealed the presence of IS10R, encoding for an active transposon commonly found in *Enterobacteriaceae* [26]. Introduction of this IS10R into the *mcr-1* gene resulted in a non-functional *mcr-1* gene. Interestingly, two identical *mcr-1* genes with IS10R duplicates were located on *IncX4*, a plasmid that has been frequently observed in combination with *mcr-1* [10, 12, 27, 28]. The *mcr-1* containing *E. coli*

belonged to ST359, this ST with a very similar antimicrobial resistance pattern is earlier described on chicken retail meat in Denmark [10]. Though we tested all morphological different Gram negative *Enterobacteriaceae* for the presence of *mcr-1*, we cannot exclude the possibly that more than one *mcr-1* containing bacterial species was present in the positive tested feces samples.

Pham Thanh *et al.* reported the first *mcr-1* positive but colistin susceptible isolate, a *Shigella sonnei*, that was based on a truncated *mcr-1* gene caused by a 22bp duplication [29]. A colistin susceptible *mcr-1* containing *E. coli* isolate with unknown cause of the susceptibility was reported in August 2016 by Liassine *et al.* [25]. Although the altered *mcr-1* gene of the *Shigella sonnei* could be re-activated by conjugation experiments resulting in a colistin resistant phenotype, the *mcr-1* gene interrupted with IS10R containing *E. coli* of this study cannot be re-activated, as upon removal of an IS transposon two remaining nucleotides would disrupt the reading frame of the gene [30]. These studies underline the importance of phenotypical confirmation after molecular screening, as respectively the *E. coli* and *Shigella sonnei* isolate showed colistin susceptibility despite the presence of *mcr-1* gene sequences that had been detected by PCR amplification.

The *mcr-1* positive *E. coli* isolate showed resistance to third generation cephalosporins due to the presence of a AmpC β-lactamase gene, *blaCMY-2*, as has previously been found by Prim *et al.* [24]. As almost all earlier studies only screened for the presence of *mcr-1* in ESBL producing isolates, the true extent of the *mcr-1* prevalence may be underestimated [10, 11, 14, 19, 31, 32].

Most likely, the kidney transplant patients acquired the *mcr-1* gene in the community, for instance by consumption of *mcr-1* containing retail meat [6, 10, 12, 13, 32]. Spread of the *mcr-1* gene in the community and successively in the hospital would pose a threat to patients developing an infection with *mcr-1* containing multidrug resistant isolates. *Enterobacteriaceae* resistant to both carbapenems and colistin by the presence of plasmid mediated *mcr-1* have already been reported [17, 31, 33-35]. Therefore, screening for and isolation of *mcr-1* containing patients should be considered. Prudence and close monitoring is necessary, especially when selective gut decontamination with colistin for ICU and hematological stem cell patients is common practice.

In conclusion, the current prevalence of *mcr-1* suggests that spread from the community into the hospital environment is low, but cannot be excluded. Furthermore the finding of a colistin susceptible, *mcr-1* containing *E. coli* underlines the importance of phenotypical confirmation after molecular screening.

Acknowledgements

Preliminary results from this study were presented at the European Congress of Clinical Microbiology and Infectious Diseases, April 9-12, 2016, Amsterdam, the Netherlands [36].

Supplementary information

S1 Table. *Mcr* real-time PCR and culture results of all 621 screened fecal samples

S2 Table. Accession numbers of the six contig containing *mcr-1* positive, colistin susceptible *E. coli.*

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Chapter 4

Spread of ESBL-producing *Escherichia coli* in nursing home residents in Ireland and the Netherlands may reflect infrastructural differences

Low MDRO rate in nursing home residents

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Chapter 4. Spread of ESBL-producing *Escherichia coli* in nursing home residents in Ireland and the Netherlands may reflect infrastructural differences

Abstract

A prevalence study in two nursing homes (the Netherlands and Ireland) found four (11%) Dutch and six (9%) Irish residents colonised with 11 extended-spectrum ß-lactamase (ESBL)-producing *Escherichia coli*, ten containing CTX-M-15. Four Dutch isolates, from three residents of the same ward belonged to *E. coli* O25:H4, sequence type (ST) 131 and were part of the same cluster type by whole genome sequencing. Four Irish residents on three different wards were colonised with an identical *E. coli* O89:H9, ST131, complex type 1478. Cross-transmission between three Irish wards may reflect differences in nursing home infrastructure specifically communal areas and multi-bedded resident rooms.

Introduction

Nursing home residents have multiple risk factors for colonisation with multidrug resistant organisms (MDRO), and are potential reservoirs for transmission [1]. Frequent contact between residents due to communal living, high frequency of healthcare contact and factors that facilitate MDRO spread such as incontinence present additional opportunities for transmission. MDRO prevalence varies considerably in nursing homes from 55 % colonisation with extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and 3 % vancomycin resistant enterococci (VRE) colonisation in Ireland [2], to 4.2 % ESBL-producing *E. coli* colonisation in the Netherlands [3]. *Clostridioides difficile* colonisation in nursing home residents also varies from 4 – 51 % [4]. We conducted a prevalence study of *C. difficile* and MDRO colonisation (specifically, VRE, ESBL and carbapenemase-producing Enterobacteriaceae (CPE)) in two nursing homes, one in the Netherlands and the other in Ireland to identify characteristics associated with carriage and risk factors for cross-transmission.

Materials and methods

Full time residents of two nursing homes under the governance of the investigators hospitals and in the investigators catchment area, one in the Netherlands and one in Ireland, were invited to participate. Written informed consent by the resident or his/her proxy was required. The nursing homes were similar to previously studied nursing homes in terms of infrastructure and resident demographics [5]. The Dutch nursing home consisted of 131 beds in eight wards varying in size (12 – 35 beds) that consisted of single en-suite rooms, except for three double rooms for couples. All wards had a separate dining area and the nursing home had a large communal recreation and physiotherapy area. The Irish nursing home consisted of 100 beds in four, identical 25-bed wards with one common recreation and dining area. Each ward consisted of a mixture of single (n=17), double (n=2) and four-bed (n=1) en-suite rooms.

Demographic and MDRO risk factor data (care load indicators, hospitalisation, antibiotics, urinary catheter use, wounds, pressure sores, previous MDRO or CDI) were collected on each consenting resident using standardized definitions [5] in February 2017 (6-17 February in the Netherlands, 6-10 February in Ireland). A corresponding

faecal specimen was also collected, stored at 4°C, processed for multidrug-resistant Enterobacteriaceae, VRE and *C. difficile* within 72 hours of arrival to the laboratory and subsequently stored at -20°C.

Ethical approval was granted by the medical ethical committee "Medisch Ethische Toetsings Commissie" of Leiden University Medical Center (no. P16.039) and the Beaumont Hospital Ethics (Medical research) committee.

Following national recommendations, Dutch faeces samples were enriched in 15ml of Tryptic Soy Broth and incubated for 18 hours at 35°C prior to plating on ChromID ESBL, VRE agar and MacConkey tobramycin agar (bioMérieux, Marcy l'Etiole, France) for 48 hours at 35°C. In Ireland, faeces were directly inoculated on identical agar plates. Isolates were identified by the BD Bruker MALDI-TOF Biotyper (Microflex, Bruker Daltonics, Bremen, Germany). Antibiotic susceptibility testing was performed by VITEK2 (The Netherlands; card N199, bioMérieux) or BD Phoenix™ automated AST system (Ireland; BD Diagnostics), using the European Committee of Antimicrobial Susceptibility Testing breakpoints. ESBL production was confirmed by a double disk method. Specimens were screened for the presence of CPE and isolates with a meropenem minimum inhibitory concentration >0.25 mg/L (Etest, bioMérieux) investigated by an in-house multiplex PCR to detect KPC, VIM, NDM, OXA-48 and IMP. *C. difficile* was detected as previously described **[6]**, and suspected colonies tested by MaldiTOF (Ireland) or an in-house GDH PCR (the Netherlands) **[6]**.

Whole genome sequence analysis (WGS) analysis was performed to further characterize ESBL-producing *E. coli* isolates from both nursing homes at GenomeScan (Leiden, the Netherlands). Genome sequences were determined using the Illumina HiSeq 4000 platform (Illumina, San Diego, CA) from DNA prepared by the QIAsymphony DSP Virus/Pathogen Midi kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. Sequence libraries were prepared using NEBNext® Ultra™ II DNA Library Prep Kit for a 150 bp paired-end sequencing. All raw sequencing data was submitted to the European Nucleotide Archive (ENA) under accession numbers ERR3151305 - ERR3151315. Core-genome Multi Locus Sequence Typing (cgMLST) was performed using SeqSphere+ software version 5.1.0 (Ridom GmbH). The number of targets for *E. coli* is 2513 with a Cluster-Alert distance of 10.

A minimum spanning tree based on the generated complex types was created in SeqSphere and expanded by uploading seven known complete genomes of *E. coli* ST131 (accession numbers: CP021179, CP021454, NC022648, CP014316, CP006784, CP010876, HG941718). The web-based tools ResFinder and RGI/CARD were used to determine antibiotic resistance genes.

Statistical analysis was performed using SPSS 23.0 and STATA SE version 15.1 (StataCorp, Texas, US). Numerical data were compared with an unpaired t-test. For categorical data, an odds ratio (OR) was calculated using logistic regression and presented with a 95% confidence interval (95% CI). For statistical comparisons, a p-value below 0.05 was considered significant.

Results

Data and a corresponding faeces specimen was collected from 37/64 (57.8%) Dutch and 67/86 (77.9%) Irish residents. (Table 1). One Dutch resident had previous VRE colonisation, whereas 27 Irish residents were previously MDRO colonised (17 methicillin resistant *Staphylococcus aureus*, seven VRE, three ESBL-producing *E. coli*). Dutch residents were less likely to have received an antibiotic and be hospitalised in the previous six months (OR 0.31, CI 0.14-0.73 and OR 0.19, CI 0.04-0.92 respectively) (Table 1).

Table 1. Socio-demographics and risk factors for multidrug resistant organism (MDRO) and *C. difficile* colonisation and infection of residents in the Dutch (NL) and Irish (IR) nursing homes.

	NL (n=37)	IR (n=67)	NL+ IR (n=104)	Odds Ratio [a] (95% CI) (NL vs IR)
Mean no. of beds/room	1.1	1.8	1.5	
Room type: Single	34 (91.9%)	44 (65.7%)	78 (75.0%)	5.92 (1.64 – 21.39)
Room type: Double	3 (8.1%)	8 (11.9%)	11 (10.6%)	0.65 (0.16 – 2.61)
Room type: Four-bed	0 (0.0%)	15 (22.4%)	15 (14.4%)	-
Mean length of residence (range)	2.1 years (0.7 - 3.8)	2.9 years (0.05 – 5.98)	2.6 years	0.036 b
Mean age (range)	84.5 years (66-95)	84.1 years (69-94)	84.2 years	0.771 b
Gender –female (%)	25 (67.6%)	43 (64.2%)	68 (65.4%)	0.86 (0.37 – 2.01)
Mobility: Ambulant	14 (37.8%)	22 (32.8%)	36 (34.6%)	1.25 (0.54 – 2.87)
Mobility: Wheelchair	23 (62.2%)	39 (58.2%)	62 (59.6%)	1.17 (0.52 – 2.69)
Mobility: Bed-ridden	0 (0.0%)	6 (9.0%)	6 (5.8%)	-
Disorientated	25 (67.6%)	53 (79.1%)	78 (75.0%)	0.55 (0.22 – 1.36)
Recentc hospitalisation	2 (5.4%)	15 (22.4%)	17 (16.3%)	0.19 (0.04 – 0.92)
Current antibiotic use	3 (8.1%)	8 (11.9%)	11 (10.6%)	0.65 (0.16 – 2.61)
Recentc antibiotic use	14 (37.8%)	44 (65.7%)	58 (55.8%)	0.31 (0.14 – 0.73)
Urinary catheter in situ	3 (8.1%)	17 (25.4%)	20 (19.2%)	0.26 (0.07 – 0.95)
Pressure sore	5 (13.5%)	2 (3.0%)	7 (6.7%)	5.08 (0.93 – 27.62)
Other wounds	13 (35.1%)	1 (1.5%)	14 (13.5%)	35.75 (4.43 – 288.14)
Incontinence: Urine	24 (64.9%)	38 (56.7%)	62 (59.6%)	1.21 (0.52 – 2.82)
Incontinence: Faeces	7 (18.9%)	43 (64.2%)	50 (48.1%)	0.11 (0.08 – 0.55)
Incontinence: Both	7 (18.9%)	33 (49.3%)	40 (38.5%)	0.21 (0.82 – 4.50)
Proton pump inhibitor use	26 (70.3%)	37 (55.2%)	63 (60.6%)	1.91 (0.82 – 4.50)

a Significance is indicated in bold. The risk factor analysis was only performed for the residents of which faeces was collected.
b For age and length of stay of the residents, differences between the two countries was calculated with an unpaired t-test. Instead of an odds ratio, the p-value is shown.
c In the previous six months
CI: Confidence Interval

Four (11%) Dutch and six (9%) Irish residents were colonised with eleven ESBL-producing *E. coli*. One Dutch resident was colonised with two different isolates. Of the Dutch isolates, four derived from three residents on ward R, were phenotypically similar on antibiotic susceptibility testing including resistance against tobramycin and ciprofloxacin. The fifth isolate from a resident on a different ward (ward L), was also resistant to gentamicin and trimethoprim/sulfamethoxazole (TMP/SMX) with intermediate resistance to tobramycin and ciprofloxacin. Of the six Irish isolates, residents were located on three different wards (wards B, C, H); five isolates were also resistant to ciprofloxacin and TMP/SMX and one resistant to gentamicin. Ten (five Dutch and five Irish) of the eleven MDRO isolates harboured CTX-M-15. No residents were colonised with CPE or *C. difficile*. No Dutch residents were colonised with VRE, in contrast to one Irish resident.

The four Dutch ESBL-producing *E. coli* isolates from ward R were typed as *E. coli* serotype O25:H4, sequence type 131. Core genome (cg) MLST analysis showed that two isolates (from two residents) had identical complex types (CT) 1172, two isolates from a third resident were closely related (CT 1480 and 1479) and belonged to the same cluster type (Fig 1). The fifth Dutch isolate from a different ward (L), was distinct (CT 1483). All six Irish isolates were typed as *E. coli* serotype O25:H4, sequence type 131. Four isolates from residents on three different wards (wards B, C, H) were closely related and belonged to CT 1478 and the same cluster type (Irish cluster, *Figure 1*). The two other Irish isolates, CT 2923 and CT 1487 were unrelated. Four of the seven epidemiologic unrelated *E. coli* ST131 from Europe (Denmark (two isolates), Germany, Austria), United States (Minneapolis two isolates), and Australia, with complex type 3100 clustered together in one cluster type *(Figure 1)*.

None of the following were significantly different for ESBL-colonised (n=10) versus ESBL-negative (n=94) residents; age (OR1.04, 95% CI 0.93 – 1.15), mean length of residence (OR 0.90 95% CI 0.61 – 1.33), previous MDRO (OR1.60 95% CI 0.18 – 15.09), residence in a single room (OR 0.46, 95% CI 0.12-1.77), recent hospitalisation or antibiotic use (OR: 0.54 95% CI 0.06 – 4.55 and OR 1.21 95% CI 0.32 – 4.58 respectively), disorientation, faecal incontinence, urinary catheter, pressure sore or other wounds (OR 0.76, 95% CI [0.18, 3.16], OR 2.55, 95% CI [0.62, 10.49], OR 1.06, 95% CI [0.21, 5.4], OR 4.45, 95% CI [0.74, 26.71], and OR 1.70, 95% CI [0.32, 9.02], respectively.

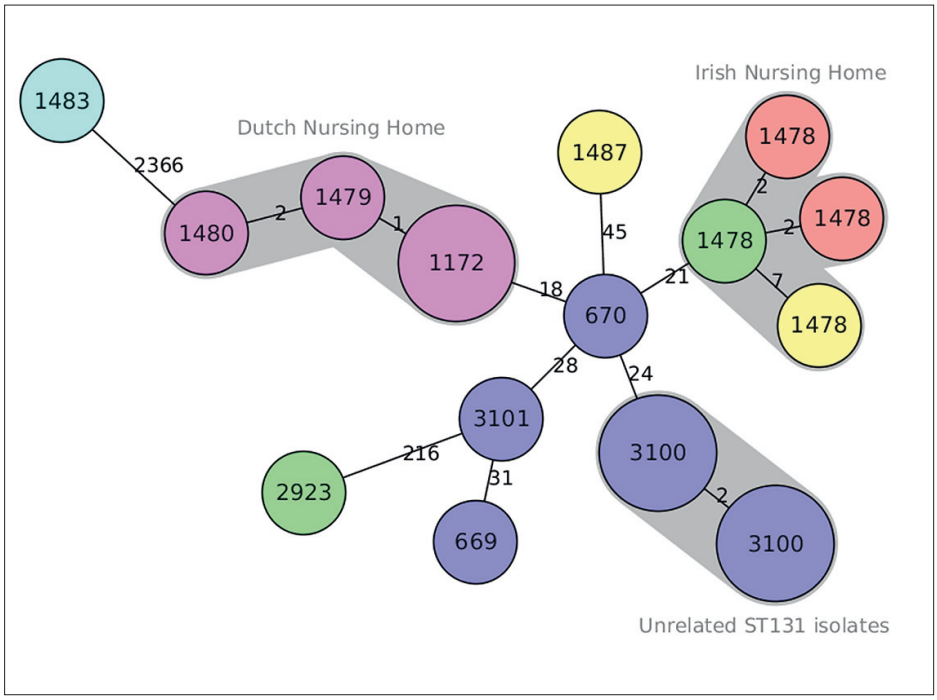

Figure 1. Minimum spanning tree of core genome MLST data of 11 ESBL-producing *E. coli* isolates

Circles: represent a core genome (cg) MLST complex type. A larger size circles represents two MDRO isolates (Complex Type 1172 and 3100), the smaller circles one isolate each. The circles are connected to the closest relative; the numbers on the connecting lines give the number of genes containing SNPs.

Colours: represent MDRO isolates from different wards in both nursing homes and the unrelated ST131 isolates. In Ireland; ward B in red, ward C in yellow, ward H in green. In the Netherlands; ward R in pink and ward L in turquoise. The unrelated ST131 isolates are coloured in blueberry blue.

Zones around the circles indicate the presence of closely related isolates belonging to the same cluster type (cluster alert distance: 10).

Discussion

The prevalence of ESBL producing *E. coli*, in Ireland (9 %), was lower than previously reported [2], whereas in the Netherland the 11% prevalence is in line with previous reports [3,7]. No resident was colonised with CPE or *C. difficile* in either country, and only one (Irish) resident was VRE colonised. Antibiotic use prevalence in both nursing homes were similar to that previously reported [5]. No association of MDRO carriage with investigated risk factors was found, which reflects the low numbers of MDRO colonised residents.

Nine of the eleven (82 %) ESBL-producing *E. coli* isolates belonged to the MLST ST131 with *CTX-M-15* as most common ESBL. The predominance of ST131 is not surprising as it is associated with and older age [8], and is frequently observed in European nursing homes [2,7]. Of the seven epidemiologically unrelated *E. coli* ST131 NCBI strains from Europe, the US and Australia, four clustered together with cgMLST in one cluster type. This further underlines the clonality of this pandemic strain.

WGS of *E. coli* isolates shows possible small-scale spread between three wards in the Irish nursing home and within one ward in the Dutch facility. There may have been more opportunities for cross-transmission in Ireland because of multi-bedded rooms and communal dining in contrast to predominance of single rooms and ward-based dining in the Netherlands. Transmission of ESBL-producing Enterobacteriaceae was higher within households than in hospitals (23 % versus 4.5 for ESBL *E. coli*, $p < 0.01$) emphasising faecal-oral transmission in ESBL epidemiology [9]. Likewise, a recent Dutch study reported co-carriage between preschool children and their parents within the same household with identical extended-spectrum cephalosporin-resistant Enterobacteriaceae, suggesting clonal transmission between children and parents within the household [10]. If transmission dynamics in nursing homes are reflective of household contact MDRO transmission, then the consequences of colonisation and initial small-scale MDRO spread could be significant. This would be compounded in nursing homes by faecal incontinence (e.g., 64 % Irish versus 19 % Dutch residents in this study) communal areas and multi-bedded resident rooms. In addition, a simulation study of MDRO transmission noted that while the daily probability of transmission in nursing homes was less than the acute hospital setting, the longer length

of resident stay (*e.g.* mean 2.6 years in our study) can facilitate cross-transmission, hence, hospital-based control efforts may not be effective in preventing nationwide outbreaks [1].

In this study, no residents were colonised with CPE or *C. difficile*, and only one Irish resident was colonised with VRE, which is in line with previous reports [2, 4], although higher *C. difficile* colonisation prevalence was reported in Ireland (10 %), albeit in a single nursing home study.

Limitations of this study include its cross-sectional design, which was chosen for pragmatic reasons, potential selection bias from inclusion of only one nursing home per country, and low resident consent and specimen collection specifically in the Netherlands, reflecting local challenges in acquiring informed consent but limiting the generalisability of findings. Specifically, the analysis of MDRO risk factors and association with MDRO colonisation was underpowered because of low numbers and the cross-sectional design limited analysis of epidemiological risk factors for colonisation, beyond associations with ward location. As data collection was based on previous European nursing home prevalence studies [5], additional data such as scores for resident independency that could impact on social contact with other residents were not collected. However, data on mobility (ambulant, wheelchair, bed-ridden) was collected as an indicator of care load with little difference between both sites. Strengths include the use of robust definitions, a standardized shared protocol, and the extensive molecular analysis. The study protocol was based on that from previous European studies [5], and similar protocols for faeces collection and laboratory processing were employed. The only difference was the use of an MDRO enrichment broth in the Netherlands, which may have resulted in a higher recovery rate. However, both countries applied the national recommended culture methods enabling a national comparison and previous Irish studies did not use an enrichment step enabling comparison [2].

In conclusion, in a nursing home prevalence study, the high abundance of risk factors did not lead to high MDRO prevalence. Core genome MLST analysis showed small-scale MDRO spread between residents of the same ward in the Netherlands and on different wards in Ireland. This may reflect differences in nursing home infra-

structure, specifically communal areas and multi-bedded resident rooms in the Irish nursing home which were not present in the Netherlands.

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Conflicts of interest

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Part II

The initiation of the Netherlands Donor Feces Bank to facilitate quality assured faecal microbiota transplantation

Chapter

5

How to: Establish and run a stool bank

Development of the Netherlands Donor Feces Bank

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Chapter 5. How to: Establish and run a stool bank

Abstract

Since 2013, several stool banks have been developed following publications reporting on clinical success of "Fecal Microbiota Transplantation" (FMT) for recurrent *Clostridium difficile* infections (CDI). However, protocols for donor screening, fecal suspension preparation and transfer of the fecal suspension differ between various countries and institutions. Moreover, no European consensus exists regarding the legislative aspects of the fecal suspension product. Internationally standardized recommendations about the above mentioned aspects have not yet been established. In 2015, the Netherlands Donor Feces Bank (NDFB) was founded with the primary aim to provide a standardized product for the treatment of patients with recurrent CDI in the Netherlands. Standard operation procedures for donor recruitment, donor selection, donor screening, and production, storage and distribution of frozen fecal suspensions for FMT were formulated. Our experience summarized in this review addresses current donor recruitment and screening, preparation of the fecal suspension, transfer of the fecal microbiota suspension and the experiences and follow-up of the patients treated with donor feces of the NDFB.

Background

Clostridium difficile, recently reclassified as *Clostridioides difficile* [1] is capable of inducing diarrheal disease (*C. difficile* infection, CDI) due to the production of secreted toxins [2]. After CDI treatment, the risk of a recurrence within eight weeks is 15–25%, which rises to 40-65% in patients with multiple recurrences [2,3]. Recurrences are associated with clinically severe diarrhoea and persistent disturbance of the colonic microbiota [4]. Fecal Microbiota Transplantation (FMT) is therefore a very effective treatment for recurrent CDI, with cure rates close to around 85% [5,6]. Large scale implementation of FMT in daily clinical practice is hampered by lack of easily available donor feces and safety concerns. A centralized stool bank can overcome these hurdles.

Aim and structure of a non-profit stool bank

The overall and primary aim of a stool bank is to provide on a (inter) national or regional level, ready to use, high-quality donor feces solutions to treat patients with recurrent or refractory CDI. Secondly, a central stool bank should enable careful monitoring of treatment outcome, side effects and long term effects of FMT. Therefore, the stool bank should preferably be facilitated by a well-equipped biobank to store an aliquot of the donor feces, and samples of all delivered fecal suspensions, to guarantee traceability in case of adverse events. A stool bank is ideally entwined with a clinical microbiological department as the expertise and equipment to perform both various screening tests, and to process fecal suspensions is already present. Since FMT is not yet an approved, treatment modality by the European Medicines Agency (EMA) or US Food and Drug Administration (FDA), commercial stool banks are not the preferred suppliers. A stool bank working group should consist of experts in the fields of Microbiology, Infectious Diseases, Gastroenterology, Biobanking, Methodology, and if donor feces is considered as a drug; Pharmacology. An overview of the currently existing donor feces banks is depicted in Table 1. Similar as to the NDFB, most of the donor banks are non-profit and primarily use FMT for treatment of patients with recurrent CDI.

Table 1. Overview of currently existing donor feces banks.

Location, founded	Legislation	Donors	Products	Indications	No.of issued products [a]	Contact address and website
Leiden University Medical Centre, The Netherlands, 2015	Allowed for CDI, no legal guideline	Healthy unrelated donors, unpaid	Fresh frozen stool samples	Recurrent/refractory CDI Pilot study for IBS Clinical trial for MDR bacteria	31	info@NDFB.nl http://www.ndfb.nl/
OpenBiome, Somerville Massachusetts, USA, 2012	Regulated as an investigational biologic, "enforcement discretion" permits use of FMT for rCDI without IND	Rigorously screened universal donors; compensated $40 per donation	Fresh frozen stool samples in 3 delivery formats: upper delivery, lower delivery and oral delivery (capsules)	CDI not responding to standard therapies Clinical trials for all other indications	23,000	Info@openbiome.org http://www.openbiome.org/
Birmingham, UK, 2015	MHRA manufacturers licence needed for clinical trial use. Special licence for CDI	Healthy unrelated donors, unpaid	Fresh frozen stool samples	Recurrent/refractory CDI	>200	PHE Public Health Laboratory Birmingham, bhs-tr.HPI@nhs.net
Portsmouth, UK, 2013	Officially under MHRA as a medicinal product	Healthy, unrelated donors, unpaid	Fresh and frozen stool samples (frozen since July 2015)	Recurrent/refractory CDI	70	fmt@porthosp.nhs.uk
Saint-Antoine Hospital, AP-HP, Paris, France, 2014	Allowed for CDI (considered as a drug). Clinical trial for other indications	Healthy related or unrelated donors, unpaid (paid for clinical trial)	Fresh frozen stool samples	Recurrent CDI Clinical trial for Crohn's disease	55	Prof. Dr. Harry Sokol, Gastroenterology Dept. Saint-Antoine hospital harry.sokol@aphp

Abbreviations; CDI: Clostridium difficile infection, IBS: Irritable Bowel Syndrome, MDR: Multi Drug Resistant, IND: Investigational New Drug, MHRA Medicines & Healthcare products Regulatory Agency, UC: Ulcerative Colitis, GvHD: Graft versus Host Disease

a (until 1-April-2017), b Commercial, social enterprise

Table 1. continued

Location, founded	Legislation	Donors	Products	Indications	No.of issued products [a]	Contact address and website
University Hospital Cologne, Germany, 2014	No legal guideline	Healthy, unrelated donors, unpaid	Frozen preparations for endoscopic application, enema or in capsules	Recurrent CDI	82	Clinical Microbiome Research Group. Dr. Maria J.G.T. Vehreschild Dept. of International Medicine, University Hospital, Cologne
Hospital Ramón y Cajal, Madrid, Spain 2016	No legal guideline	Healthy related or unrelated donors, unpaid	Fresh frozen stool samples	Recurrent CDI, in principle local patients only	13	Dr. López-Sanromán, Gastroenterology, Hospital Ramon Y Cajal, 28034 Madrid
Medical University Graz, Austria, 2012	Allowed for CDI based on national guideline Other indications need ethics committee board approval	Healthy related and unrelated volunteers. Clinical trials compensated with €50.-/donation	Fresh and frozen faecal samples ready to use for lower GI- endoscopy	Recurrent CDI Severe CDI Idiopathic colitis Colitis in critical ill patients Clinical trials for UC, IBS, GvHD.	400	Theodor Escherich Laboratory for Microbiome Research, www.medunigraz.at
Asia Microbiotab Bank, Hong Kong, 2016	No legal guideline	Healthy unrelated donors, paid	Frozen processed microbiota samples (no fresh or whole stool samples available clinically)	Recurrent CDI Primary CDI Clinical trial for IBS, IBD and MDR bacteria	In process, to be determined	health@asiabiobank.com www.asiabiobank.com

Abbreviations; CDI: Clostridium difficile infection, IBS: Irritable Bowel Syndrome, MDR: Multi Drug Resistant, IND: Investigational New Drug, MHRA Medicines & Healthcare products Regulatory Agency, UC: Ulcerative Colitis, GvHD: Graft versus Host Disease

a (until 1-April-2017), b Commercial, social enterprise

Legislation of a donor feces bank

There is still considerable confusion about the regulatory aspects of FMT [7-10]. The FDA dictates that adequate informed consent must be obtained before use of FMT products [11]. In the European Union (EU), a standardized policy is lacking and each member state is allowed to have its own policy. In the Netherlands, FMT is currently regarded as an unclassified treatment approach, which is allowed (if applied safely), for patients with recurrent CDI, or in the context of an approved investigational study protocol.

Although FMT appears a typical transplantation product to most experts in the field [12], it does not fulfil the criteria for guidance by the EU tissue and cell transplantation act, because the cellular component of FMT appears not to be the active substance. Furthermore, human excretions are excluded by the US act for tissue and cell transplantations. As a consequence, several European countries are considering donor feces as a drug (Table 1), which has major regulatory implications negatively influencing future availability and pricing of donor stool solutions for FMT. Application as a drug has the consequence that the proposed drug would have to be identical in active ingredient, dosage form, route of administration, quality, and performance characteristics. However, the complexity of the microbial community in stool and the variability across stool samples makes it impossible to guarantee the contents from batch to batch. Furthermore, it would have the consequence of putting fecal material for use in FMT under the jurisdiction of hospital pharmacies, requiring storage of the fecal product in the pharmacy itself. In this regard, common sense and consultation of the experts in the field may hopefully result in adjustment of the EU law in concordance with the rapid scientific developments, enabling a future status of donor feces as transplantation product.

How to recruit donors?

Historically, FMT donors were conveniently selected among close relatives and friends of patients with the underlying idea that they would have at least a partially shared microbiome, increasing the chances of success [13], and limiting the risk of pathogen transmission [13,14]. However, later evidence showed that FMT with donor

feces from unrelated donors was as effective [5, 15]. This finding provided an opportunity for a better standardized, safer, faster and cheaper method of donor selection, screening and fecal suspension preparation.

The NDFB acquired many potentially interested donors after announcing the opening of the first Dutch stool bank via local and national media (e.g. paper, national news). One of several options for recruitment of feces donors are amongst established blood donors, as this has the advantage of previously screened, healthy and motivated volunteers. An important difference in the donor recruitment in the Netherlands and most other European countries (except Germany) compared to the USA is that it is prohibited to offer a paid reimbursement for blood (or stool) donations. This prohibition, is in line with the blood donating advice of the World Health Organization which states that the safest blood donors are voluntary, non-remunerated donors [16]. As it is important to limit the time between defecation and delivery of the feces, to preserve as much anaerobes, donors should be recruited in the near proximity of stool banks, such as non-health care workers of the hospital and personnel of companies in the neighbourhood.

Donor screening by questionnaire and interview

All potential donors are extensively screened by a questionnaire and a personal interview concerning risk factors for transmissible diseases and factors influencing the intestinal microbiota (Table 2). The NDFB has applied an arbitrary age limit of 18 to 50 years, assuming that above the age of 50 years a significant increase of comorbidities with a less stable microbiota can be present [17]. A body mass index (BMI) > 25 kg/m^2 is also an exclusion criterion, since obesity may also be associated with a specific microbiota composition [18]. Moreover, one case-report, and an experimental animal study suggesting new-onset obesity after infusion of donor feces of an overweight donor has been reported [19, 20]. Any other gastrointestinal disorder (e.g. irritable bowel syndrome (IBS), Crohn's disease and ulcerative colitis) also qualifies as an exclusion criterion of donation [21]. Other exclusion criteria that have been shown to be related to aberrant microbiota composition are depicted in box 1 [22]. The list of exclusion criteria will probably expand in the future when other conditions are found to be associated with an altered microbiota composition.

Box 1. Aim and exclusion criteria of the donor screening by questionnaire.

Aim:
Risk assessment of fecal- and/or blood transmitted diseases and illnesses associated with a disturbed microbiota.

Exclusion criteria:
Age <18 or ≥ 50, BMI <18.5 or > 25 [19, 20], high risk fecal- and or blood transmittable diseases, recent antibiotic use (<6 months) [23, 24], gastrointestinal complaints (for example diarrhoea, obstipation or irritable bowel like symptoms) [25-27], recent travel to endemic areas of gastrointestinal pathogens, (first degree relative with) inflammatory bowel disease [28], GI malignancy [29], first degree relative with a GI malignancy < 60 years, substantial comorbidity, various medication, autism [22, 30, 31], auto-immune disorders [32], neurological disease [33, 34]

Donor screening by laboratory tests

An extensive laboratory analysis should be performed to identify potential pathogens transmissible by fecal transfusion. An overview of all tests performed by the NDFB is shown in Table 2. The pathogens included in the blood-screening program correspond with the screening protocols for blood donors and are generally agreed upon between the different stool banks [14, 15, 35-39]. However, screening protocols for detection of specific microorganisms in the intestinal tract differ between stool banks, and evolve with time and new insights, since there is no consensus guideline. This applies for example to the screening for the presence of multidrug resistant (MDR) organisms, including ESBL- and carbapenemase-producing bacteria, vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus*. Screening for the (asymptomatic) presence of rotavirus is not routinely performed by stool banks, but since rotavirus is frequently found in asymptomatic donors, especially in winter, we included this in our protocol [38, 40]. Adenovirus type 40/41, Sapovirus and Astrovirus are associated with mild gastro-intestinal diarrhoea and are therefore also screened [41]. Enterovirus and Parechovirus are usually asymptomatic but can cause skin disease (and-foot-and-mouth disease), pleurodynia, myocarditis and meningitis [42, 43]. Adenovirus non-40/41 can cause myocarditis [44]. In addition, feces is screened for Hepatitis E, which is frequently found in asymptomatic (blood)

donors [45]. To prevent transmission and development of systemic infections, potential donors are screened with PCRs for all the above mentioned viruses (see also Table 2 for the total list of pathogens).

Table 2. Donor screening by laboratory screening of feces and serum.

When donors pass the questionnaire, feces is first screened for the presence of Dientamoeba fragilis and *Blastocystis* hominis. When negative, other pathogens are investigated, after which screening of serum is performed.

• **Laboratory screening serum**	**Laboratory screening feces**
• Hepatitis A (IgM + IgG) • Hepatitis B (HBsAg + anti-Hbcore) • Hepatitis C (anti-HCV) • Hepatitis E (IgM + IgG) • HIV (anti-HIV, type 1 and 2) • Lues; *Treponema pallidum* (Ig) • Cytomegalovirus (IgM + IgG) • Epstein Barr Virus (IgM + IgG • *Strongyloïdes* (IgG1/IgG4) [a]	• *Clostridium difficile* (PCR) • *Helicobacter pylori* (antigen test) • Bacterial gastro-enteritis: (PCR, followed by culture)): *Salmonella* spp. *Campylobacter* spp., *Campylobacter jejuni, C. coli, Shigella* spp., *Yersinia enterocolitica* and *Y. pseudotuberculosis, Aeromonas* spp., *Plesiomonas shigelloides*, and Shiga Toxin producing *E.coli* • Antibiotic resistant bacteria (culture); ESBL and/or carbapenemase producing bacteria, vancomycin resistant enterococci and methicillin resistant *Staphylococcus aureus* • Viral pathogens (PCR): Norovirus serotype I+II, Astrovirus, Sapovirus, Rotavirus, Adenovirus 40/41, Adenovirus non-40/41, Enterovirus, Parechovirus, Hepatitis E • Parasites (PCR): *Giardia lamblia, Entamoeba histolytica, Cryptosporidium parvum and C. hominis, Microsporidium spp, Strongyloïdes* [a] • Microscopy for ova, cysts and larvae [46]: for example: *Blastocystis hominis*
Questionnaire: One day before donation of feces Stool frequency/pattern, general health, use of antibiotics, travel history, sexual behaviour	

a If travel history to Middle and South America, Africa or Asia

The significance of *Dientamoeba fragilis and Blastocystis hominis* as enteropathogens is less clear [47-50]. *D. fragilis* and *B. hominis* are commonly found in fecal samples of both symptomatic and asymptomatic individuals [50, 51]. Prevalence varies considerably depending on geographic location, the group studied, and diagnostic methods used [47]. The cell wall of *B. hominis* is fragile and disrupts easily; storage of microscopically positive stool samples in 10 % glycerol at -80°C results in complete lysis and negative microscopy after the samples are thawed and reinvestigated (unpublished observation). Despite the uncertainty of *B. hominis* and *D. fragilis* pathogenicity, colonisation may be considered an indicator of a suboptimal microbiota composition [52]. Therefore, positive individuals are excluded from donorship for NDFB.

The serostatus of the donor is determined for Epstein-Barr virus (EBV) and Cytomegalo virus (CMV). Immunocompromised patients will be matched accordingly for safety reasons. However, the risk of transmission is not established and we await the results of ongoing study regarding the risk of CMV transmission due to FMT (TRANSDECMV Clin Trial Gov: NCT02694484).

Approximately two months after the initial screening, a new donor sample of feces and blood are screened again, using similar tests as applied at entry of the program (see Table 2), except for CMV and EBV which are repeated once a year (in case of a negative serostatus. After a successful second screening, the donor fecal suspensions collected until two weeks prior to the second screening are released for patient treatment. This quarantine period minimizes the risk for transmissible diseases.

Collection, preparation and storage of donor feces suspensions

It is generally believed that a high viability of bacteria in stools increases the chance of successful FMT. Since the majority of fecal bacteria are anaerobic, feces needs to be processed within six hours after defecation **[5, 6]**. To prevent environmental contamination, feces is collected by the donor in a fecal container (for instance Fecotainer™). For suspension, approximately 60 gram of donor feces is used based on the data of a systematic review suggesting a decreased cure rate with < 50 gram **[53]**. The feces is homogenized with saline using a mortar and pestle, whereas some laboratories use a commercial blender **[15, 37]**. Disadvantages of blenders are difficulties with appropriate sterilisation and aerosolization of the feces. A metal sieve (mesh 300µm) is used to remove undigested food fragments. The fecal suspension is then concentrated by centrifugation (15 minutes, 6000g) **[37]** and glycerol is added as cryoprotectant to a final concentration of 10 % in a total end volume of 200 ml. A recent study showed that frozen fecal suspension is equally effective as a fresh fecal suspension for the treatment of CDI **[54]**. This allows stool samples to be stored at -80°C for a longer period of time until the donor has been retested prior to actual use of the donor feces. Clinical success of frozen suspensions is reported until five to six months of storage at -80°C, but could be much longer, in theory. Like OpenBiome, the NDFB uses a storing period of two years.

How to apply safety measures and include quality controls

At the Leiden University Medical Center (LUMC), storage of the FMT suspensions is accommodated by the certified centralized biobanking facility in a specific -80°C freezer with connected alarm notification to guarantee a continuous registration of the storage. In addition, the biobanking facility uses a dedicated biobanking information and management system (BIMS SampleNavigator®) for coding, registration, tracking and tracing of the bio samples. FMT suspensions, in combination with a small portion of the original feces and a 2 cc portion of the FMT suspension, are stored under a unique donor code with a successive suffix number for donation time and date for retrospective quality assessment. Information on the FMT suspension labels includes donor code, suspension number, production and expiration date, volume, and storage temperature instruction. Distribution of the FMT upon granted request by the NDFB is provided by dry-ice shipment through a certified Biologistic Courier service. Registration in a BIMS-related database for the shipped FMT suspensions, including recipient institution and requestor information, is provided in order to be able to perform biovigilance tracing in cases of adverse events.

An important aim of the NDFB is to recognize complications of FMT. Therefore, systematic follow-up of both patients and donors is performed with signed informed consents. The NDFB collects recipients' feces and clinical data on the day of FMT and approximately three weeks after the procedure. Furthermore, clinical information including abdominal complaints, development of diarrhea and adverse events (e.g. nausea, bloating, abdominal pain, belching, vomiting) is collected. No systematic long-term follow-up has been scheduled yet to register development of auto-immune diseases, malignancies and other potentially microbiome-associated syndromes both in donors and patients. However, all feces and serum samples have been stored in the biobank and remain available for analysis.

How to determine eligibility of patients with recurrent *Clostridium difficile* infection for FMT

Since the effectiveness of FMT has only been recognized by the authorities for recurrent CDI, it is extremely important to diagnose recurrent CDI both with the presence of clinical symptoms, and positive microbiological tests. Therefore, written requests for FMT treatment with a standardized form are evaluated by at least two clinical members of the NDFB board to determine eligibility of the patient. It is required that patients have a laboratory documented episode of recurrent CDI following at least one course of adequate CDI antibiotic therapy (≥10 days 125 mg vancomycin QID; ≥10 days metronidazole 500 mg TID; 10 days 200 mg fidaxomicin BID). Recurrent CDI is defined as the re-appearance of diarrhoea (≥3 unformed stools per 24 hours for two consecutive days; or ≥8 unformed stools per 48 hours) within eight weeks after cessation of antibiotic therapy in combination with a positive diagnostic test for *C. difficile*. We strongly recommend a two-stage testing algorithm, as recently advised by the *C. difficile* ESCMID diagnostic guideline **[55]**. In particular, a positive test for the presence free toxins in feces samples (e.g. by EIA) is a prerequisite, especially for patients with comorbidity of the intestinal tract, such as inflammatory bowel disease (IBD). If laboratories only use a PCR to detect toxin genes of *C. difficile*, we advise to send a fresh feces sample to a reference laboratory for toxin detection, since *C. difficile* (spores) can persist after successful treatment and may reflect colonisation.

For a first recurrence of CDI, it is advised to first treat the patient with another course of antibiotics. Fidaxomicin could be considered because of potentially relapse reducing effect due to its narrow antibiotic spectrum **[56]**. In general, FMT is advised in patients with multiple recurrences. However, in some cases of severe, therapy refractory CDI, FMT could be considered for a first recurrence **[39, 57]**. A recently completed study suggests that intravenously administered humanised monoclonal antibodies against *C. difficile* toxin B (bezlotoxumab) protects against (multiple) recurrent CDI. However, it is yet unclear which patients really benefit from this very expensive treatment strategy **[58]**.

Pregnancy, severe food allergy, and antibiotic usage other than for *C. difficile* at the day of expected infusion are exclusion criteria for FMT treatment. Although, recently,

the first case report of successful and safe FMT in a pregnant patient has been published [59]. All potential risks, benefits, logistics, and procedural details are discussed with the patient by the treating physician.

What is the procedure of FMT?

If the patient is eligible for treatment with FMT, donor feces suspension is transported to the referring hospital on dry ice. Prior to transplantation, the feces suspension is thawed (overnight in a 4°C refrigerator or during five hours at room temperature), based on literature and our expert opinion [15, 54, 60]. The donor feces suspension may be kept at room temperature for up to three hours or refrigerated at 4°C for up to six hours. Samples should never to be re-frozen, because freeze-thaw cycles may compromise stability and efficacy of the sample, possibly due to loss of viability. To eradicate vegetative cells of *C. difficile*, prior to FMT (until one day before the procedure), patients receive vancomycin (125-250 mg QID) for a minimum of four days, followed by two litres of bowel lavage one day prior to FMT [5]. Whether bowel lavage can be excluded from the protocol is currently a matter of discussion, since recent reports have shown similar efficacy for FMT without bowel lavage [61-63]. The treating physician is advised to avoid antibiotics in FMT patients during the first month after FMT unless strictly necessary, and preferably as small as possible. FMT is generally performed by infusion of a donor feces solution through a gastric or duodenal tube [5], colonoscope [6], or enema [54]. All infusion routes have advantages and disadvantages, and in every patient the ideal method should be evaluated. The FMT procedure can be performed by the treating physician and does not justify standard referral to a specialised centre. Physicians are instructed how to perform FMT, and if necessary, FMT training sessions are offered. In the Netherlands, FMT via duodenal tube is preferred because it is generally well tolerated by patients, and is less invasive compared to colonoscopy, especially in an inflamed bowel as with severe CDI [5, 64, 65]. On the day of FMT treatment, a duodenal tube is placed through duodenoscopy, radiological guided, or with use of an electromagnetic imaging system (e.g. Cortrak TM). The thawed feces solution of approximately 200 ml is slowly infused through the duodenal tube with a 50 cc syringe, at a rate of 10 cc/minute, after which the tube is flushed with 50 ml tap water. Thirty minutes after FMT, the duodenal tube is removed and patients are monitored for two hours. If FMT through a duodenal tube is

contra-indicated (i.e. due to a hampered bowel passage or higher risk of aspiration), FMT is performed via colonoscopy. We generally do not advice enemas, because of the need of repeated FMT's to achieve a high cure rate with enemas **[66]**.

NDFB experience during May-January 2017

In March 2016, the opening of NDFB was reported in various local and national newspapers and broadcasted in radio and television programs, accompanied by an invitation for volunteers to register as donor. Subsequently, 165 volunteers registered and informed by email about the procedure and were requested to complete an online questionnaire. After this evaluation only 21 potential donors (12.7%) were screened for the presence of transmissible diseases (Table 3). Nine (5.5% of initial responders) volunteers passed the screening and were invited to donate. This percentage is low, though in line with earlier reports on donor screening **[40, 67-69]**. The fecal suspensions were quarantined for two months after which the donors were re-screened. Two volunteers had to temporarily stop donating for three months because of an episode of acute diarrhoea. Four donors did not pass a re-screening: two carried *B. hominis*, one an ESBL positive *E. coli* (exclusion for at least 6 months) and one donor a rotavirus (indication for re-screening of the previous donated samples and exclusion for 2 weeks); this underlines the importance of a quarantine period. As a substantial portion of donors only donates temporarily, donor recruitment is a continuous process.

In May 2016, the first FMT with a donor feces suspension of the NDFB was performed. In the first nine months after its opening, 31 feces suspensions to 18 different hospitals throughout the Netherlands have been distributed for treatment with FMT. We noticed a cure rate of 84%, which is in line with the earlier reported randomized controlled trails **[5, 6]**.

Table 3. Experiences of the NDFB with donor screening

Potential donors	Action	Exclusion reasons [a]	Excluded (n)	Suitable donors [b] (n)
165	Request of information by email	62% age > 50 years, 26% unable to deliver feces < 2 hour after defecation, 6% BMI > 25, 6% other	94 (57%)	71 (43%)
71	Extended questionnaire	17.2% age > 50 years, 27.1% BMI > 25, 14.3% (history of) depression, 8.5% comorbidity/medicine use, 7.1% profession of health care worker [c], 7.1% inability to deliver feces < 2 hour, 7.1% (close relative with) IBD, 4.3% anorexia, 2.9% recent use of antibiotics [d], 2.9% autism, 2.9% (risk factors for) colon carcinoma [e], 2.9% profession with frequent travelling, 2.9% abundant flatulence	50 (70.4%)	21 (12.7%)
21	First feces screening	42.9% *D. fragilis*, 4.8% *D. fragilis* and *B. hominis*, 4.8% *D. fragilis* and *C. jejuni*, 4.8% *E. histolytica* [f]	11 (52.3%)	10 (6.1%)
10	Serum screening	None	0 (0%)	10 (6.1%)
10	Repeated feces screening	20% *B. hominis*, 10% ESBL *E.coli*, 30% donor withdrawal (after 0, 2 and 6 months) Temporarily excluded: acute diarrhoea (for 3 months), rotavirus carriership (for 2 weeks)	6 (60%)	4 (2.4%)

a Some volunteers have multiple exclusion criteria
b 1 minus cumulative proportion of excluded donors
c Higher risk of temporary carriership of pathogens
d Antibiotic use in the previous six months
e Close relative with colon carcinoma, onset below age of 60
f Treated, included as donor six months later

Business plan

In the Netherlands, disease entities are reimbursed regardless of the given treatment (e.g. for recurrent CDI; vancomycin or fidaxomicin or FMT) when the patient is treated in daycare. A business case to calculate the break-even point of producing safe feces samples for FMT was determined for the NDFB. We differentiated between (i) recruitment, screening and selecting of suitable donors (ii) donation of feces by donors and periodic rescreening, (iii) assessment of eligibility of patients' demand for FMT (iv) supply of a safe fecal suspension, and (v) post-treatment monitoring. The costs covering involved hospital staff (medical, technical, administrative, advisory), laboratory

tests, storage and bio-banking amounts to a unit cost per patient to be treated (including 10 % re-treatment in case of initial non-response) of €899 in case of 100 patients yearly, dropping to €785 in case of 400 patients yearly to account for economies of scale.

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Chapter 6

Feces microbiota transplantation for *Clostridioides difficile* infection

Four years' experience of the Netherlands Donor Feces Bank

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Chapter 6. Feces microbiota transplantation for *Clostridioides difficile* infection: four years' experience of the Netherlands Donor Feces Bank

Abstract

Background: **The Netherlands Donor Feces Bank (NDFB) provides standardized ready-to-use donor fecal suspensions for fecal microbiota transplantation (FMT) treatment of patients with recurrent *Clostridioides difficile* infection (rCDI).**

Objective: **Evaluation of safety, feasibility and outcome of FMT facilitated by a national stool bank.**

Methods: **Observational cohort study of donors and recipients of fecal suspensions; assessment of donor screening and patient selection performed by an expert panel of medical microbiologists, gastroenterologists and infectious disease specialists; and patient outcome evaluated at different timepoints post-FMT.**

Results: **Donors: Of 871 volunteers who registered as potential feces donor, 16 (2 %) became active donors. Nine donors stopped or were excluded after a mean donation period of 5.7 months. Patients: Between May 2016 and August 2019, 47 (27 %) of the 176 requested FMTs were deemed not indicated by the expert panel. In total, 129 rCDI patients were treated with 143 FMTs in 40 different hospitals across the Netherlands. The cure rate at two months after a single infusion was 89 % (107/120). Of 84 patients, long-term follow-up (median 42 weeks) was available and sustained cure was achieved in 61 (73 %). Early CDI relapses (within two months after FMT) and late recurrences (after more than two months) occurred more frequently in patients who received non-CDI antibiotics within three weeks post-FMT and in moderately to severely immunocompromised patients. Of 21 patients with post-FMT CDI, 14 were cured with anti-CDI antibiotics and seven with a second FMT. No FMT-related serious adverse events were observed, but gastrointestinal complaints (nausea, abdominal pain or diarrhoea) persisted in 32 % of the treated patients at long-term follow-up.**

Conclusion: Fecal suspensions provided by a centralized stool bank, supported by a multidisciplinary expert team, resulted in effective, appropriate and safe application of FMT for rCDI.

Established knowledge on this subject	Significant and/or new findings of this study?
» Fecal microbiota transplantation (FMT) is an established therapy for multiple recurrent *Clostridioides difficile* infection (rCDI).	» FMT that is facilitated by a national stool bank, is efficacious, safe and appropriately used.
» Only a small percentage of potential donors is eligible after careful selection and screening.	» Consultation by a multidisciplinary FMT-expert team results in appropriate use of FMT.
» Centralized stool banks provide an opportunity for quality improvement of FMT	» Post-FMT CDI relapse can be treated with antibiotics directed against CDI, even if these were ineffective prior to FMT in those patients.
	» Feacal suspensions for rCDI treatment can be stored at -80°C for up to two years, without loss of effectivity.

Introduction

Fecal microbiota transplantation (FMT) is a very effective treatment for recurrent *Clostridioides difficile* infection (rCDI). In recent years it has been implemented worldwide as effective rescue therapy with cure rates of approximately 85 % [1-4]. Transplanting fecal microbiota of a healthy donor with the aim to restore a patient's perturbed microbiota appears also promising for several other disorders, such as ulcerative colitis and hepatic encephalopathy [5, 6]. Careful donor screening is required, minimizing the risk of pathogen transfer or an impaired microbiota composition potentially predisposing

for disease. With the emergence of FMT as a new treatment approach, stool banks are needed to provide ready-to-use donor fecal suspensions that are produced in a standardized way [7]. Significant advantages of centralized donor screening and production of donor fecal suspensions are the possibilities to provide quality assurance, and appropriate monitoring of potential yet unknown adverse events [7].

At present, stool banks operating at an institutional level exists in several countries, and national operating stool banks are active in the US, the Netherlands and England [8-12]. In 2015, the Netherlands donor feces bank (NDFB) was founded as a non-profit national stool bank. In addition to providing fecal suspensions, the NDFB provides advice on the diagnosis, treatment and follow-up of recurrent or severe CDI by an FMT-expert panel of medical microbiologists, gastroenterologists and infectious disease specialists. The NDFB expert panel evaluates each request for FMT [10].

The aim of the current evaluation report was to describe the results of donor screening and the outcome of FMT performed for rCDI facilitated by the NDFB, and under guidance of its expert panel. In addition, donor-, patient- and fecal suspension-specific factors underlying FMT treatment failure are addressed.

Material and methods

Study design

This is a prospective, observational cohort study describing the results of feces donor screening and patient outcome after FMT from the first performed donor screening in January 2016, and FMT in May 2016, until August 2019.

Screening & selection of donors

The NDFB recruits healthy, unrelated volunteers who can supply stool to the to the microbiology laboratory within two hours after defecation. The procedure of donor recruitment, screening by questionnaire, interview and laboratory testing was described before [10], and is summarized in supplementary Table S1.

Processing and storage of fecal suspensions

The NDFB uses standardized procedures for collection, preparation and storage of donor fecal suspensions [10]. In short, sixty grams of donor feces is used for the preparation of one fecal suspension. Storage at -80°C is accommodated by a certified biobanking facility. At the NDFB, the maximum shelf-life has been (arbitrarily) determined as two years.

FMT consultation and treatment

Requests for fecal suspensions are submitted to the NDFB by treating physicians using a standardized form (www.ndfb.nl). The request is evaluated by at least three medical specialists (a gastroenterologist, medical microbiologist and infectious disease specialist) of the NDFB expert panel. The indication for FMT is assessed, the diagnosis of rCDI is verified, and the feasibility and safety of FMT for the individual patient is considered. Patients with at least two recurrent CDI episodes or severe and therapy refractory CDI are eligible. CDI is defined as diarrhoea (≥3 unformed stools per 24 hours for two consecutive days; or ≥8 unformed stools per 48 hours), in combination with a positive diagnostic test for *C. difficile* and absence of another more likely cause of diarrhoea. To differentiate between infection and asymptomatic colonization, a two-stage testing algorithm is recommended [13]. In particular, presence of free *C. difficile* toxins is a prerequisite for patients with gastro-intestinal comorbidity. Severe CDI is defined by the presence of severe colitis or a complicated course, with systemic toxin effects and shock that may result in ICU admission or colectomy [14].

If a patient is eligible for FMT, a donor fecal suspension is transported to the requesting hospital on dry ice and thawed according precise instructions [10]. In general, prior to FMT, patients receive vancomycin (125-250 mg QID) for a minimum of four days until 24 hours pre-FMT. For duodenal delivery, two litres of KleanPrep (bowel lavage) is prescribed one day prior to FMT [10]. Treating physicians are instructed how to perform FMT. The thawed fecal suspension is infused through a duodenal tube, at an advised rate of 10cc/minute. If FMT through a duodenal tube is considered unsafe or contra-indicated (i.e. due to a hampered bowel passage or increased aspiration risk), infusion via colonoscopy is advised. After infusion of

the donor feces, patients are monitored for two hours [1,10]. Antibiotic stewardship to protect the microbiota post-FMT is advocated to prevent a relapse of CDI after FMT [10,15,16].

Follow-up

At each FMT treatment, the patient and treating physician receive information on potential adverse events and are advised to contact the NDFB if such an event occurs. Treating physicians are advised to plan a routine follow-up visit at three weeks post-FMT and patients are requested to complete a questionnaire. Patients are routinely approached by an NDFB employee by telephone two months after FMT, and for the present evaluation report also at a later time-point between January 2019 and August 2019 (19 to 143 weeks) post-FMT for long-term follow-up. Information about recurrence, hospital admission, possible FMT-related adverse events and antibiotic use is collected. We defined early relapse as a CDI episode within two months following FMT [14], whereas a CDI episode after two months post-FMT was regarded as late recurrence. We defined cure as resolution of all CDI symptoms, and no CDI relapse within three weeks (primary cure), two months (cure at two months) or long-term follow-up (sustained cure). We categorized the relationship between adverse events and FMT as follows: definitely related, probably related, possibly related and unrelated to FMT [17].

Statistical analysis

The statistical analysis was performed using SPSS 23.0 statistical software. Continuous data are presented as mean (range), or median in case of a skewed distribution. Possible associations between FMT treatment outcome and patient, fecal suspension or donor characteristics were tested by a Pearson's Chi-squared test, or Fisher's exact test where appropriate. An odds ratio was calculated using logistic regression and presented with a 95% confidence interval [95% CI]. For ordinal data, a linear-by-linear association test was used. Kaplan-Meier curves and log-rank tests were performed to assess CDI-free survival. A two-tailed significance level of $p<0.05$ was considered statistically significant. Missing data and patients lost-to follow-up were mentioned but data was not corrected for this.

Results

Donor selection & screening

Since the initiation of the NDFB, 871 volunteers registered as potential feces donor. After receiving information about donor requirements, 603 withdrew and 268 completed an online questionnaire (Table 1). Based on the questionnaire, 83 (31%) donors were invited for an interview, followed by microbiological testing. After evaluation of the interviews, screening and rescreening of feces and serum, only 16 volunteers were eligible as feces donor, which is 6% of volunteers completing the questionnaire and 2% of all initially interested individuals (Table 1). Of these 16 active donors, 10 (63%) were female, the mean age was 33 (range 24-57) years, and the mean BMI was 22.4 (range 19.6-24.8) kg/m^2. Asymptomatic, transient carriage of potential pathogens was occasionally found at re-screening (multidrug resistant organism (MDRO): n=4, norovirus: n=2, rotavirus: n=1, sapovirus: n=1, parechovirus: n=1, *Salmonella* species: n=1, or *Dientamoeba fragilis*: n=1). Nearly all active donors experienced one or more transient episodes with upper respiratory complaints, diarrhoea, temporary change of defecation pattern, or antibiotic use, for which donations were temporarily stopped. Nine of the 16 (56%) donors stopped or were excluded after a mean period of 5.7 months (range 1-14 months). Reasons for discontinuation were persistent carriage of potential pathogens during repeated testing (*Blastocystis* species: n=2, MDRO: n=1, or *D. fragilis* n=1) or a too heavy burden of required time and logistics (n=5).

Table 1. Results of the donor selection and screening process

Donors (%)	Action	Excluded (%)	Exclusion reasons †
871	Request for more information by donor	603 (69%)	52% (n=311) withdrawal after reading additional information 22% (n=132) unable to deliver feces < 2 hrs after defecation 20% (n=121) age > 50 years ‡ 8% (n=49) increased risk disturbed microbiota (bowel complains, medication use, comorbidity, depression, BMI > 25 m^2/kg, etc.), 5% (n=29) other
268 (100%) ↓	Donor fills-out an extended questionnaire	185 (69%)	22% (n=41) comorbidity/medication use 22% (n=40) BMI < 18.5 or > 25 m^2/kg 18% (n=33) (history of) depression 15% (n=28) profession of health care worker § 14% (n=25) age > 50 years‡ 14% (n=25) bowel complaints 12% (n=22) inability to deliver feces < 2 hours 10% (n=19) withdrawal after completing questionnaire 6% (n=12) (close relative with) IBD 5% (n=9) frequent travelling 4% (n=7) risk factor for colon carcinoma ¶ 3% (n=6) high risk sexual behaviour 7% (n=13) other
83 (31%) ↓	Interview	17 (20%)	65% (n=11) donors withdrawal or failure to deliver feces <2h once a week 35% (n=6) donors excluded based on interview (IBS complaints, comorbidity, psychological evaluation, patient contact, atopy)
66 (25%) ↓	Feces¥ screening	47 (71%)	89% (n=42) *Dientamoeba fragilis* 15% (n=7) MDRO 9% (n=4) *Blastocystis* sp. 4% (n=2) *Helicobacter pylori* 2% (n=1) *Campylobacter jejuni* 2% (n=1) *Entamoeba histolytica*
22[A] (8%) ↓	Serum screening	0 (0%)	None
22 ∫ (8%) ↓ 16 (6%)	First rescreening & donor withdrawal Active donor	6 (27%)	Exclusion of quarantined donor suspensions: 83% (n=5) difficulty to implement donation in daily practice, 17% (n=1) MDRO and refusal to perform rescreening

Abbreviations: MDRO: multidrug resistant organism, IBS: Irritable Bowel Syndrome

† Some volunteers had multiple exclusion criteria, exclusion is displayed as the percentage of total excluded donors as result of a particular screening step.

‡ From September 2018 changed to 55 years, or 60 years with negative colon carcinoma screening.

§ Higher risk of temporary carriership of pathogens

¶ Close relative with colon carcinoma with an onset below the age of 50

¥ Screening algorithm used: first screening includes: Dientamoeba fragilis, microscopy for Blastocystis sp., MDRO and Helicobacter pylori screening, if negative, then additional tests are performed (Table S1)

∫ 3 donors were excluded at first screening, successfully decolonized of MDRO, D. fragilis or E. histolytica, and they subsequently continued the donor screening program.

FMT consultation

Since May 2016, 176 FMT requests for treatment of (r)CDI patients were reviewed by the expert panel. Of these requests, 47 (27%) were deemed not indicated. The most frequent reason for rejection was *C. difficile* carriership in combination with diarrhoea due to inflammatory bowel disease (IBD) or another, unknown cause. Detailed results of the evaluation of FMT requests are listed in Table 2.

Table 2. Results of the evaluation of FMT requests by the multidisciplinary FMT expert panel

FMT decision	Number of requests	
FMT request rejected by NDFB expert panel	**47/176 (27%)**	
Reasons of rejection of the 47 FMT requests:		
C. difficile carriership & diarrhoea due to other cause;	30 (64%)	
» Diarrhoea with unknown cause	–	18
» Diarrhoea due to IBD	–	12
Anti-CDI antibiotics advised instead of FMT;	11 (23%)	
» First, mild recurrence	–	7
» New CDI infection (too long interval between CDI episodes)	–	4
Long-term antibiotic use/elective operation	3 (6%)	
Withdrawal of FMT request after observed antibiotic treatment effect, by treating physician or patient	3 (6%)	
Request for FMT approved by NDFB expert panel	**129/176 (73%)**	
FMT indication[†]		
» Multiple recurrent CDI	125 (97%)	
» Severe, therapy refractory CDI	3 (2%)	
» Refractory CDI	1 (1%)	

Abbreviations: CDI: Clostridioides difficile infection, IBD: Inflammatory Bowel Disease

[†] 143 FMTs performed in 129 patients. Ten patients received multiple FMTs; 9 patients for treatment of a post-FMT CDI relapse (7 patients cured with a single repeat FMT, 1 patients cured with 2 repeat FMTs, 1 patient cured with antibiotics after a repeat FMT) and 1 patient received sequential FMT treatment for severe, therapy refractory CDI (in total 6 FMTs; 3 FMTs for a first episode and 3 FMTs for the relapse).

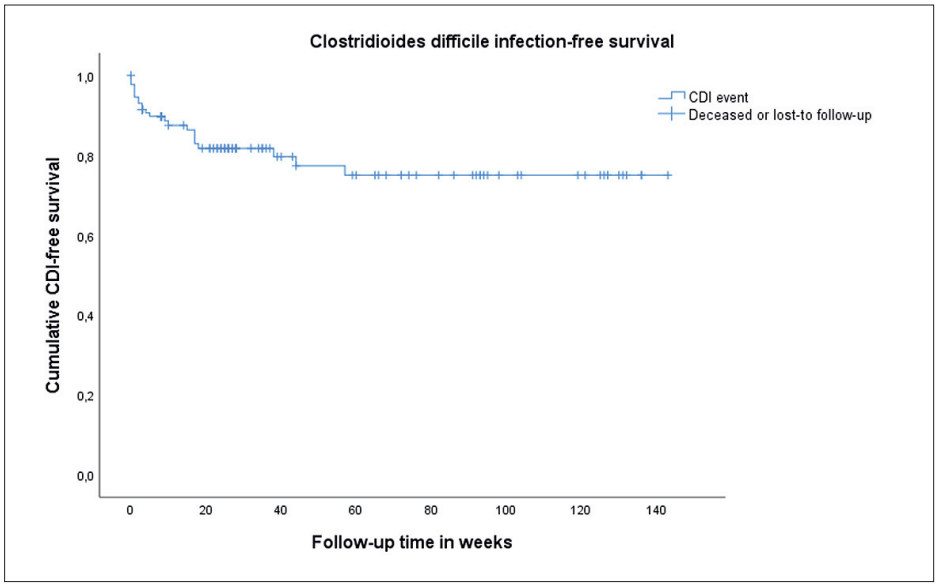
FMT treatment

In total, 129 patients with CDI were treated with 143 FMTs in 40 different hospitals throughout the Netherlands. Suspensions obtained from 12 of the 16 approved donors were used. The mean age of the patients was 69.9 years (range 2-96), and 60% were female. Patients suffered from a mean of 4.2 (range 1-10) CDI episodes before FMT

was considered. Most patients had rCDI (Table 2). Four patients received an FMT for a first episode of severe, therapy-refractory CDI, of whom one received multiple FMTs (six in total). The majority of FMTs (127/143, 89 %) were infused through a duodenal tube. FMT via the lower gastrointestinal route was performed by colonoscopy because of motility disorders (n=4), an already planned colonoscopy to rule out IBD (n=8); or sigmoidoscopy because of an ileus due to severe CDI (n=4).

Outcome of FMT treatment

Follow-up data were available for 128 of 129 patients at three weeks, and 120 patients at two months after FMT. Three patients (2 %) died within three weeks due to causes unrelated to the FMT. The primary cure rate at three weeks after a single FMT infusion was 91 % (117/128). Cure at two months post-FMT was 89 % (107/120). Thirteen



Number of patients at risk for CDI after FMT:

Time (weeks)	0	3	8	20	40	60	80	100	120	140
Patients (number)	129	119	105	70	37	31	23	14	11	1

Figure 1. Kaplan Meier curve of the *Clostridioides difficile* infection (FMT failure, early relapse or late recurrence)-free survival post-FMT.

patients suffered from an early relapse at a median of 1 week (range 0-5 weeks) post-FMT. Of the 129 FMT treated patients; 11 (9 %) were deceased by the time of long-term follow-up, 34 (26 %) were lost to follow-up. From 84 (65 %) patients, long-term follow-up was available with a median of 42 weeks (range 19-143 weeks, interquartile range 26-97 weeks). Ten patients developed a late CDI recurrence after a median of 17 weeks (9-57 weeks) post-FMT. Thus, sustained cure was achieved in 61 of 84 (73 %) patients still alive at long-term follow-up. *Figure 1* shows the CDI free survival over time. The 23 patients suffering from post-FMT CDI had symptoms of diarrhoea, either in combination with a positive toxin EIA (14/23, 61 %), PCR (5/23, 22 %), or were diagnosed with unclear methods but with clinical response to vancomycin treatment (4/23, 18 %). Most patients experiencing CDI post-FMT eventually successfully cured (21/23), either by antibiotics alone (14/21, 67 %; received fidaxomicin, 4 vancomycin, 1 metronidazole, and 1 fidaxomicin), or by a second FMT (7/21, 33 %; of whom one patient needed a third FMT). In two patients CDI treatment was not initiated, the patients died of an underlying disease with concurrent development of CDI.

Risk factors for post-FMT CDI

Patients with an early CDI relapse post-FMT had more often received non-CDI antibiotics during the first three weeks after FMT compared to patients without relapse (39 % versus 15 %) (Table 3). Antibiotic use preceded a late CDI recurrence in 80 % of patients. Nonetheless, antibiotic use shortly after FMT was still a significant predictor of all CDI episodes post-FMT (both early AND late) (40 % (8/20) compared to 15 % (14/95), *p-value* 0.001, *Figure S1*). In addition, early CDI relapses were observed more frequently in patients who were moderately (3/23) to severely (2/3) immunocompromised (Table 3). A trend was observed towards more CDI (early relapse or late recurrence) post-FMT in immunocompromised (31 %, 8/26) versus immunocompetent patients (15 %, 15/101) (*p-value* 0.054, *Figure S2*). No other patient or fecal suspension characteristic significantly differed between those who relapsed and those cured *(Table 3)*. Importantly, a longer processing time of fecal suspensions (mean 168 minutes, range 65–355 minutes) or longer storage time at -80°C (mean 269 days, range 34–730 days, 30/129 were stored >1 year) did not negatively influence the success rate of FMT. Donor selection did not influence the outcome of FMT; no differences between donors could be detected (p-value 0.10, individual donor data in Table S2).

Table 3. Patient, donor and fecal suspension risk factors for CDI relapse within two months after FMT

Characteristic	Patients with relapse within two months post-FMT	Patients cured at two months post-FMT	Results statistical analyses (OR [95% CI], p-value)
Patient sex (female)	77% (10/13)	58% (67/116)	OR 2.4 [0.6-9.3], p-value 0.24
Donor sex (female)	54% (7/13)	50% (58/116)	OR 1.2 [0.4-3.7], p-value 0.79
Donor – patient sex mismatch	39% (5/13)	47% (54/116)	OR 0.7 [0.2-2.3], p-value 0.58
Patient's age (at FMT)	69 years (41-96)	70 years (2-92)	p-value 0.76
Donor's age (at donation)	36 years (24 – 46)	35 years (24-46)	p-value 0.82
Lower gastro-intestinal infusion of FMT (sigmo- or colonoscopy)	23% (3/13)	8% (9/116)	OR 3.6 [0.8-15.3], p-value 0.10
Mean processing time of the fecal suspension (defecation to freezer)	163 minutes	168 minutes	p-value 0.73
Mean storage time of the fecal suspension (at -80°C)	214 days	275 days	p-value 0.27
Severe CDI as indication for FMT	8% (1/13)	2% (2/116)	OR 4.8 [0.4-56.3], p-value 0.28
Prior CDI relapses, before FMT is performed	2.6 (13)	2.8 (114)	p-value 0.69
PPI use	61% (8/13)	51% (55/108)	OR 1.5 [0.5-5.0], p-value 0.47
Comorbidity of IBD	8% (1/13)	11% (13/114)	OR 0.7 [0.1-5.4], p-value 1.0
Severe kidney comorbidity: dialysis or kidney transplantation	8% (1/13)	9% (24/112)	OR 1.0 [0.1-8.2], p-value 1.0
rUTI in medical history	0% (0/13)	8% (9/113)	p-value 0.60a
Use of non-CDI antibiotics in between the prior CDI episodes	46% (6/13)	38% (43/113)	OR 1.4 [0.4-4.4], p-value 0.57
Immunocompromised †			
– Not	61% (8/13)	82% (93/114)	**p-value 0.01**
– Moderate	23% (3/13)	18% (20/114)	
– Severe	15% (2/13)	1% (1/114)	
Hypervirulent clade‡	25% (2/8)	20% (13/65)	OR 1.3 [0.2-7.4], p-value 0.66
Post-FMT hospitalization for non-CDI indications post-FMT ¶	23% (3/13)	14% (14/102)	OR 1.9 [0.5-7.7], p-value 0.41
Post-FMT infection (other than CDI) ¶	15% (2/13)	17% (17/102)	OR 0.9 [0.2-4.5], p-value 1.00
Post-FMT antibiotic use (non-CDI indications) ¶	39% (5/13)	15% (15/102)	OR 3.6 [1.0-12.6], **p-value 0.03**

Abbreviations: CDI: *Clostridioides difficile* infection, FMT: Fecal Microbiota Transplantation, PPI: proton pump inhibitor, IBD: Inflammatory Bowel Disease, OR: Odds ratio, rUTI: recurrent urinary tract infection, rCDI: recurrent *Clostridioides difficile* infection

† Immunocompromised classified as: not, moderate or severe. Patients are regarded as severely immunocompromised when: neutropenic, (scheduled or received last 100 days) an allogenic stem cell transplantation, active Graft-versus-host-disease requiring immunosuppressive agents, and moderately immunocompromised when: having <200 CD4 T-cells/µl, prolonged use of corticosteroids at a mean dose of 0.3mg/kg/d of prednisone equivalent for >3 weeks, treatment with other recognized T-cell immunosuppressants during the last 90 days or have an inherited severe immunodeficiency.

‡ Hypervirulent clade RT027 (016, 019, 0247, 036, 075, 111, 112, 153, 156, 176, 208, 273) and clade RT078 (033, 045, 066, 078, 126, 127).

¶ In the first 3 weeks post-FMT.

Patients: Follow-up of adverse events

On the day of FMT, 66% (62/94) of patients had mild, transient gastro-intestinal complaints (Table 4). At three weeks and at long-term follow-up, a subset of patients still reported abdominal pain (both 21%) and diarrhoea (27% and 33%, respectively). The self-rated defecation pattern after FMT compared to the pre-existent defecation pattern (before the CDI episodes) had improved in 16% at 3 weeks, and in 38% at long-term follow-up (Table 4).

Table 4. Gastro-intestinal complaints post-FMT

Gastro-intestinal complaint	Day of FMT †	1-week post FMT †	3-weeks post FMT †	LTFU ‡
Nausea (% yes)	20% (19/94)	14% (13/96)	11% (11/97)	18% (13/73)
Abdominal pain (% yes)	33% (31/93)	28% (27/97)	21% (21/98)	21% (15/71)
Diarrhoea (% yes)	52% (48/93)	30% (29/97)	27% (26/97)	33% (24/73)
Self-rated defecation pattern (post-FMT vs before CDI episode)				
Improved	n/a	n/a	16% (13/80)	38% (25/65)
Similar	n/a	n/a	68% (54/80)	46% (30/65)
Deteriorated	n/a	n/a	16% (13/80)	15% (10/65)

Abbreviations
FMT: fecal microbiota transplantation, LTFU: Long-term follow-up, n/a: not applicable, rCDI: recurrent *Clostridioides difficile* infection

† A questionnaire is filled in by the patient or treating physician at regular follow-up 3 to 4 weeks post-FMT
‡ LTFU: long-term follow-up (median 42 weeks, range 19-143 weeks)

No definitely or probably related serious adverse events were reported. Five (5/128, 4%) FMT (procedure)-related adverse events were observed (Table 5). Regurgitation of donor feces occurred in four patients shortly after duodenal infusion of the fecal suspension (Table 5). During the first three weeks after FMT, 23% (26/115) of the patients were admitted to the hospital or had prolonged hospitalization, of which nine (8%) for possibly FMT-related indications (Table 5). The most frequently observed infections after FMT were urinary tract infection (UTI) (8%, 9/115) or pneumonia (5%, 6/115). The majority of patients suffering of these infections had known predisposing factors for UTI or pneumonia (Table 5).

Table 5. (Serious) adverse events within three weeks after fecal microbiota transplantation.

Description adverse event			Number of patients
Definitively or probably related to FMT	SAE	None	0% (0/128)
	AE	Procedure related AE's	4% (5/128)
		» Regurgitation, no aspiration, patient successfully treated	– 4
		» Sore throat after placing duodenal tube	– 1
Possibly related to FMT	SAE	Hospitalization within 3 weeks post-FMT due to:	8% (9/115)
		» Lower respiratory tract infection (causing pathogen unknown) †	– 5
		» Urinary tract infection (causing pathogen unknown) ‡	– 3
		» Diarrhoea (non-CDI)	– 1
	AE	Gastro-intestinal (see Table 4)	11 - 52%
		Infections	
		» Urinary tract infection (causing pathogen unknown) ‡	– 5
		» Urinary and lower respiratory tract infection†	– 1
		Other	
		» Fever	– 1
		» Possible flare IBD (ulcerative colitis), uncertain if it was pre-existent	– 1
Unrelated to FMT	SAE	Hospitalization (or prolonged hospitalization) within 3 weeks post-FMT	15% (17/115)
		» Lower respiratory tract infection (COPD exacerbation due to Moraxella catarrhalis and RSV infection)	– 2
		» CDI relapse	– 3
		» Related to pre-existent comorbidity (elective surgery dialysis shunt, complications knee prosthesis, perforated diverticulitis, hyponatremia with tongue carcinoma, GvHD after allogenic stem cell transplantation (already existing), diarrhoea due to chemotherapy)	– 6
		» Death within 3 weeks post-FMT due to comorbidity (tongue carcinoma/hypo-natremia, sepsis due to pneumonia, comorbidity GvHD lung after allo-SCT)	– 3
		» Infection with Yersinia pseudotuberculosis post-FMT, donor suspension tested negative (with both PCR and culture (cold enrichment-broth)	– 1
		» Perforated diverticulitis, in retrospect already present before FMT (upper GI delivery)	– 1
		» CVA	– 1
	AE	Infections	
		Otitis, infection of toe, phlegmon groin	– 3

Abbreviations:
COPD: chronic obstructive pulmonary disease, CVA: cerebro vascular accident, GI: gastrointestinal, GvHD: graft versus host disease, PCR: polymerase chain reaction, RSV: respiratory syncytial virus, SCT: stem cell transplantation.

† Four patients (80 %, 4/5) developing a pneumonia had a medical history of either COPD, asthma or lung fibrosis.

‡ Four patients (44 %, 4/9) had known predisposing factors for UTI (medical history of pyelonephritis, diabetes type II and benign prostate hypertrophy, Sachse urethrotomy or Bricker bladder).

Discussion

During the four years since its establishment, the NDFB has evaluated over 175 FMT requests and provided standardized FMT to almost 130 patients affected by rCDI. A high cure rate of nearly 90% at two months after FMT and a sustained cure rate over 70% at 42 weeks post-FMT was observed.

The cure rate of FMT facilitated by the NDFB appears high compared to the 76% cure rate reported in a recent meta-analysis of single FMTs for rCDI [4]. This may be explained by the stringent criteria for diagnosis and treatment applied by our FMT expert panel [13]. This expert panel discusses the indication for FMT and provides advice during treatment and follow-up of the patients. We rejected a quarter of FMT requests, mainly because the diarrhoea was attributed to another cause that coincided with *C. difficile* carriership. Thus, consultation might prevent inappropriate use of FMT and increases the clinical benefits and cost-effectiveness. Our observation is similar to a previous report from an FMT-expert centre, which showed that 25% of patients referred for FMT did not have confirmed rCDI [18]. In particular, new onset or persistent activity of IBD appears to be a diagnostic pitfall [18,19]. In addition, non-responsiveness to anti-CDI antibiotics seems to point to an alternative diagnosis rather than therapy-refractory CDI in most patients. In fact, only four of our 129 patients were deemed to suffer from therapy-refractory by the expert-panel.

A subgroup of rCDI patients remains vulnerable for CDI after FMT, as 9% (10/107) of initially cured patients developed a late CDI recurrence. Of this group, 80% had used antibiotics preceding the recurrence, in contrast to only 39% of patients with an early relapse. This indicates that antibiotic use after FMT should be limited as much as possible for a prolonged period. It also emphasizes that a long follow-up after FMT is mandatory to assess the long-term efficacy of FMT. The majority (61%) of early relapses was not preceded by antibiotics, indicating that other factors also contribute to FMT failures, such as an immunocompetence. Other studies have identified Charlson comorbidity index [20], the severity of CDI [21,22], previous (CDI) hospitalisation [22,23], inpatient status [22], surgery [23], female sex [23] and older age [24] to predict recurrence after FMT. We did not recognize donor-related factors contributing to FMT outcome, confirming previous reports [25-27]. The majority of patients with post-FMT CDI were cured with antibiotic treatment, suggesting that this should be considered a different

entity compared to the antibiotic resistant episodes prior to FMT. This could be explained as an FMT mediated gut microbiota reset, which renders patient less susceptible to rCDI after treatment with antibiotics alone.

In our experience, duodenal delivery of donor feces was highly effective and certainly not inferior to delivery by colonoscopy, although this report was not designed as a study to compare routes of delivery. Duodenal FMT has a small risk of regurgitation. To prevent this, we currently advise slow infusion of the fecal suspension in the duodenum (10 cc/min), room temperature of the suspension to avoid cold shock, and colonoscopic delivery in case of possible bowel dysmotility. After introduction of these precautions, regurgitation was no longer recognized. We did not observe FMT-related serious adverse events. Several patients developed a UTI (9/115) or pneumonia (6/115) after FMT. This might be explained by existing predisposing factors in most patients, although a relation with FMT cannot be fully excluded. Interestingly, it has been suggested that the incidence of UTI could decline after FMT due to a reduced abundance of Enterobacterales in the gut **[28]**. About 21 %-33 % of patients suffered from abdominal complaints at follow-up. This is in line with a previous report, in which no FMT-attributable factors could be identified **[29]**. Remarkably, at long-term follow-up, the self-rated defecation pattern improved (38%), or had stayed unchanged (46 %) in most of our patients, compared to the period before the first CDI episode, suggesting that gastro-intestinal symptoms after FMT could be related to post-infectious complaints and pre-existent comorbidity. Post-infectious irritable bowel complaints after CDI were also reported in 4 to 25 % of patients not given FMT **[30]**.

We observed a low two months mortality rate of 3 % (3/120) after FMT, which is lower than the 30-day mortality rate of primary CDI in the Netherlands (9 % overall mortality) **[31]**. The mortality of 12 % at long-term follow-up (median 42 weeks) is lower than observed in two other FMT cohorts (20 % at weeks 30 and 48 post-FMT) **[32, 33]**.

A strength of our evaluation report is the structural follow-up of donors and patients of a complete stool bank cohort, with use of standardised questionnaires and over a long period of time post-FMT. Several studies report on retrospective analyses of only specific patient groups treated with stool bank FMT-suspensions without structural long-term follow-up. In one of the largest retrospective studies, 307 of 528 (39 %) rCDI patients were successfully contacted, a sustained cure of 76% at 34 months follow-up was

observed [33]. Our high sustained cure rate is confirms this observation. A limitation of our report is that 26% patients were lost to long-term follow-up, and late recurrent CDI may be overestimated as these were actively reported to the NDFB by the local physicians. This is supported by the fact that no unreported recurrences were detected with the follow-up questionnaires. Another limitation, also related to the setting of a national stool bank, is the lack of nation-wide uniform microbiological testing, which may have influenced the process of consultation and the outcome of the treatment.

The risk of infectious complications after FMT depends on appropriate donor screening. This may even be more important for severely immunocompromised patients, as suggested by the cases where transfer of MDRO by FMT in neutropenic patients resulted in sepsis and death [34]. Only 2% of potential donors were eventually eligible after extensive selection and screening. This is comparable to the donor qualification rate of 3% of a large US stool bank [8]. Others reported higher donor acceptance rates of 10-31% [35-37]. Unfortunately, donor exclusion criteria and screening-protocols are heterogeneous and often incomplete [38], underlining the need for standardization of donor screening. After initial donor acceptance, a quarter of our donors were excluded at the first quarantine screening. In addition, over half of the active donors stopped donating after six months for logistic reasons or persistent colonization by a potential pathogen. A high dropout rate was also observed in Canada; four of five approved donors were excluded during the quarantine period due to travel or acute gastro-enteritis [39]. This demonstrates the need for a quarantine period and targeted screening on indication before fecal suspensions can be used safely. Although the identification of potential pathogens such as MDRO, norovirus, or rotavirus in asymptomatic, active donors is rare, the NDFB performs complete microbiological screening of the dedicated fecal suspension when the recipient is severely immunocompromised. In the future, donor selection will be even more challenging if specific donor characteristics are required for FMT treatment for indications such as ulcerative colitis or hepatic encephalopathy [25]. In this regard, the finding that fecal suspensions with a shelf life of two years at -80°C are safe and evenly efficacious for treatment of rCDI is encouraging.

In conclusion, the use of strict donor selection criteria, standardized processing and storage of FMT suspensions, and consultation by a multidisciplinary FMT-expert team, as provided by a professional stool bank, results in safe and efficacious application of

FMT for rCDI. With the increasing number of reports pointing to potential beneficial effects of FMT in patients with a variety of gastrointestinal and extra-intestinal disorders, a growing demand of FMT can be expected in the near future. Initially, experimental studies will have to be performed in a controlled setting. However, for routine clinical practice, standardised preparation, quality control and careful and long-term monitoring of outcomes and adverse events, stool banks are required. We encourage FMT-centres and stool banks to utilize a multidisciplinary FMT-experts team to fill a currently existing gap, and ensure a safe and controlled application of FMT.

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Declaration of conflicting interest

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Ethics approval

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's Human Research Committee, and was approved on 16 December 2015 by the medical ethics committee of the Leiden University Medical Center (P15.154).

Informed consent

Both patients and donors provided written informed consent for collection and analysis of stool samples and clinical data.

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Supplementary material

Table S1. Donor selection and screening

Exclusion criteria by questionnaire:

Age < 18 or ≥ 55a, BMI < 18.5 or > 25 [40, 41], high risk fecal- and or blood transmittable diseases, recent antibiotic use (<6 months) [42, 43], gastrointestinal complaints (for example diarrhoea, obstipation or irritable bowel like symptoms) [44-46], recent travel to endemic areas of gastrointestinal pathogens, (first degree relative with) inflammatory bowel disease [47], known systemic infection, liver diseases like hepatic encephalopathy or Non Alcoholic Fatty Liver Disease [5, 48], History of cancer, including GI malignancy or polyposis [49], first degree relative with a GI malignancy < 60 years or family history of genetically-driven cancer, metabolic syndrome [50], substantial comorbidity, chronic medication use [51], autism [52-54], auto-immune disorders [55], neurological/neurodegenerative disease [56, 57], atopic diseases [58]

Laboratory screening serum	Laboratory screening feces
Hepatitis A (IgM + IgG)[b]	*Clostridioides difficile* (PCR)
Hepatitis B (HBsAg + anti-Hbcore)	*Helicobacter pylori* (antigen test)
Hepatitis C (anti-HCV)	Bacterial gastro-enteritis: (PCR): *Salmonella* spp. *Campylobacter* spp., *Campylobacter jejuni, C. coli, Shigella* spp., *Yersinia enterocolitica* and *Y. pseudotuberculosis*, *Aeromonas* spp., *Plesiomonas shigelloides*, and Shiga Toxin producing *E.coli*
Hepatitis E (IgM + IgG)[b]	
HIV (anti-HIV, type 1 and 2)	
Lues; *Treponema pallidum* (Ig)	Antibiotic resistant bacteria (culture); ESBL and/or carbapenemase producing bacteria, aminoglycoside AND quinolone resistant Enterobacterales, vancomycin resistant Enterococci and methicillin resistant *Staphylococcus aureus*
Cytomegalovirus (IgM + IgG)[b]	
Epstein Barr Virus (IgM + IgG)[b]	Viral pathogens (PCR): Norovirus serotype I+II, Astrovirus, Sapovirus, Rotavirus, Adenovirus 40/41, Adenovirus non-40/41, Enterovirus, Parechovirus,
HTLV[c]	
Strongyloïdes (IgG1/IgG4)[d]	Parasites (PCR): *Giardia lamblia, Entamoeba histolytica, Cryptosporidium parvum and C. hominis, Microsporidium spp, Cystoisospora belli, Cyclospora cayetanensis. Strongyloïdes* [d]
	Microscopy for ova, cysts and larvae [59]: for example: *Blastocystis* sp.

Questionnaire recent health status: One day before donation of feces

Stool frequency/pattern, general health, use of antibiotics, travel history, sexual behaviour

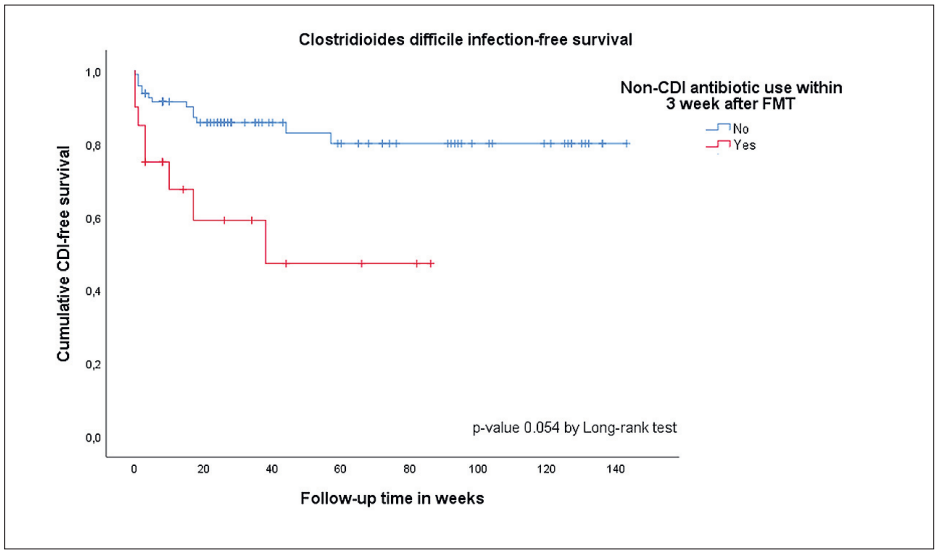
Donor screening by questionnaire, when donors pass the questionnaire, laboratory screening of feces will follow. Feces is first screened for the presence of *Dientamoeba fragilis* and *Blastocystis* sp. When negative, other pathogens are investigated, after which screening of serum is performed. If a donor is suitable for donation, before every donation a questionnaire about the recent health status should be filled in.

[a] Or 60 years when no colon cancer is detected during the national colon cancer screening programme.
[b] In case of rescreening, only repeat when prior sero-negative, to detect seroconversion and subsequent potential transmission via feces.
[c] In case of rescreening only when travelled outside Europe.
[d] In case of rescreening only when travelled to Middle and South America, Africa or Asia.

Table S2. FMT success rate individual donors

Donor	Early relapse rate (CDI <2 months post-FMT)*	Late recurrence rate (CDI >2 months post-FMT)*
1.	11.8% (2/17)	5.9% (1/17)
2.	0% (0/3)	0% (1/3)
3.	0% (0/4)	0% (0/4)
4.	25% (1/4)	0% (0/4)
5.	16% (4/25)	16% (4/25)
6.	0% (0/22)	13.6% (3/22)
7.	11.1% (2/18)	0% (0/18)
8	0% (0/3)	0% (0/3)
9.	0% (0/4)	0% (0/4)
10.	9.1% (2/22)	4.5% (1/22)
11.	0% (0/4)	0% (0/4)
12.	66.7% (2/3)	0% (0/3)

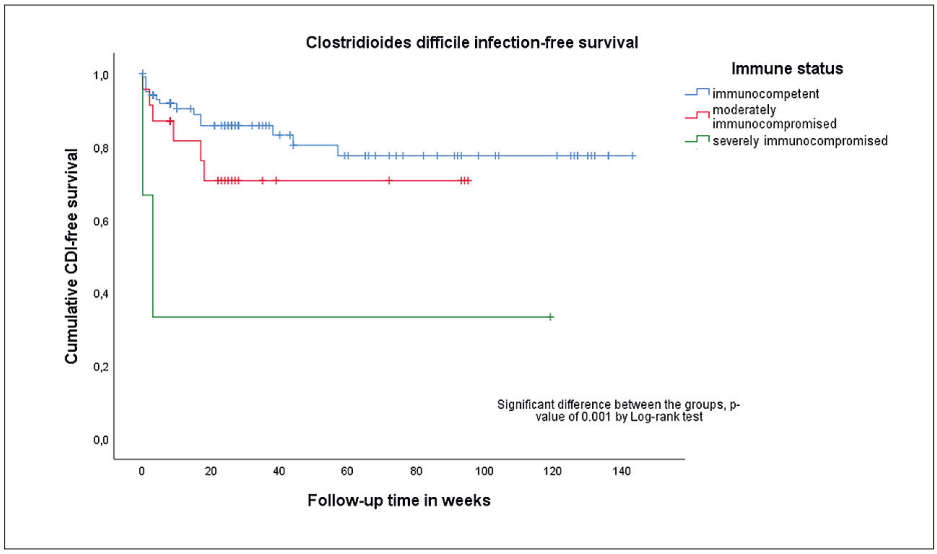
* Number of patients with an early relapse/late recurrence who received a faecal suspension of a specific donor/ total number of faecal suspensions of that donor used.



Number of patients at risk for CDI after FMT:

Time (weeks)	0	3	8	20	40	60	80	100	120	140
Antibiotic use	95	89	83	59	32	27	20	14	11	1
No antibiotic use	20	17	13	7	4	3	2	0	0	0

Figure S1. Kaplan Meier curve of *Clostridioides difficile* infection (FMT failure, early relapse or late recurrence)-free survival post-FMT of patients receiving (red line) and not-receiving (blue line) antibiotics for other indications than CDI within three weeks post-FMT.



Number of patients at risk for CDI after FMT:

Time (weeks)	0	3	8	20	40	60	80	100	120	140
Immunocompetent	101	94	82	56	32	26	19	13	11	1
Moderately immunocompromised	23	21	13	13	4	4	3	0	0	0
Severely immunocompromised	3	2	1	1	1	1	1	1	0	0

Figure S2. Kaplan Meier curve of *Clostridioides difficile* infection (FMT failure, early relapse or late recurrence)-free survival post-FMT of immunocompetent (blue line) versus moderately (red line) or severely (green line) immunocompromised patients.

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Chapter 7

Human transmission of *Blastocystis* by Fecal Microbiota Transplantation

without development of gastrointestinal symptoms in recipients

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Chapter 7. Human transmission of *Blastocystis* by Fecal Microbiota Transplantation without development of gastrointestinal symptoms in recipients

Abstract

Background: **Patients with multiple recurrent *Clostridioides difficile* infections (rCDI) are treated with fecal microbiota transplantation (FMT) provided by healthy donors. *Blastocystis* colonization of donors is considered an exclusion criterion, whereas its pathogenicity is still under debate.**

Methods: **The introduction of molecular screening for *Blastocystis* sp. at our stool bank identified two donors with prior negative microscopy but positive PCR. Potential transmission of *Blastocystis sp.* to patients was assessed on 16 fecal patient samples, pre- and post-FMT, by PCR and subtype (ST) analysis. In addition, clinical outcome for treatment of rCDI (n=31), as well as development of gastrointestinal symptoms was assessed.**

Results: **There was one donor carried *Blastocystis* ST1, the other contained ST3. All patients tested negative for *Blastocystis* prior to FMT. With a median diagnosis at 20.5 days after FMT, 8 of 16 (50 %) patients developed intestinal colonization with *Blastocystis*, with identical ST-sequences as their respective donors. *Blastocystis* containing fecal suspensions were used to treat 31 rCDI patients, with a FMT success rate of 84 %. This success rate was not statistically different from patients transferred with *Blastocystis* sp. negative donor feces (93 %, 76/82). Patients transferred with *Blastocystis* sp. positive donor feces did not report any significant difference in bowel complaints in the first week, after 3 weeks and the months following FMT.**

Conclusions: **We demonstrated the first transmission of *Blastocystis* ST1 and ST3 from donor to patients by FMT. This did not result in gastrointestinal symptomatology or have any significant effect on rCDI treatment outcome.**

Key points

Transmission of *Blastocystis* by Fecal Microbiota Transplantation from colonized donors occurred in 50 % of treated patients. Transfer did not result in development of gastrointestinal symptoms or affect the outcome of the FMT treatment in patients with recurrent *Clostridioides difficile* infections.

Introduction

Blastocystis is a genus of common unicellular intestinal parasite in humans and animals which belongs to the stramenopiles, one of the eight major phylogenetic groups of eukaryotes. It is a diverse genus comprising of 17 characterized lineages, the so-called subtypes (ST1 – ST17), of which nine have been reported to occur in the human gastrointestinal tract [1, 2]. *Blastocystis* sp. carriage is very common but varies globally from 0.5 % in Japan, to 100 % in Senegal and 30-50 % in Europe [3-6].

The pathogenicity of *Blastocystis* sp. is uncertain, and in general it is considered an innocent parasite [7]. The presumed entero-pathogenicity is based on anecdotal case reports and retrospective reviews and is mainly tested in animal models [8,9]. The symptoms attributed to this organism include nausea, anorexia, abdominal pain, flatulence and acute or chronic diarrhea [8]. However, outbreaks have never been reported and a human challenge model has not been applied. An association of *Blastocystis* sp. with irritable bowel syndrome (IBS) was suggested [10,11], but could not be confirmed in two large cohort studies [4,12]. Interestingly, *Blastocystis* sp. is found to be less prevalent in patients with inflammatory bowel disease (IBD), a disorder which is associated with a reduced diversity of the gut microbiota [4,13,14], and asymptomatic *Blastocystis* sp. carriers tend to have a more diverse microbiota [4,15-20]. These observations could indicate that the presence of *Blastocystis* sp. may reflect a more healthy and diverse state of the gut microbiota.

Patients with multiple recurrent *Clostridioides difficile* infection (rCDI) are treated with fecal microbiota transplantation (FMT) prepared with feces of healthy donors. Carriership of *Blastocystis* sp. by healthy donors is considered an exclusion criterion for donation by several stool banks, including the NDFB [21-26], resulting in considerable exclusion of donors (30-50 %). It is questionable whether this is justified. Therefore,

knowledge about potential side effects and treatment success of co-transplantation of *Blastocystis* sp. with FMT is warranted. This study reports the co-transmission of *Blastocystis* sp. from donor to patient, and the influence on the outcome and health of rCDI patients receiving FMT.

Methods

Donors and donor fecal suspensions for FMT

The Netherlands Donor Feces Bank (NDFB) is located within the Department of Medical Microbiology at the Leiden University Medical Center (LUMC) and started with treatment of patients with multiple rCDI with FMTs in 2016 [21]. All donors of the NDFB are healthy individuals between the age of 18 and 50, with normal weight (BMI 18.5 – 25) and no relevant medical history or medication use. All donors are extensively screened and rescreened for disorders associated with a perturbed microbiota and potential transmissible infectious diseases [21].

The NDFB uses standardized procedures for collection, preparation and storage of donor fecal suspensions as described earlier [21]. In short, donors deliver stool at the NDFB within two hours after defecation. Sixty grams of donor feces is used to prepare one fecal suspension. The feces was homogenized with sterile saline with use of mortar and pestle, sieved, centrifuged until an end volume of 200ml (containing 10 % glycerol). Two cc of the final fecal suspension, and two grams of original donor stool are separately aliquoted and stored as quality control. The fecal suspensions are stored within six hours following defecation. Storage is accommodated by a certified centralized biobanking facility in a dedicated -80 °C freezer with connected alarm notification and biobanking information and management system (BIMS SampleNavigator®).

Patient selection and treatment

Requests for FMT in rCDI patients are carefully evaluated by the working group of the NDFB. Upon approval, the NDFB facilitates FMT by providing ready-to-use fecal suspensions for treatment at the local hospital as previously described [21]. Patients are preferably pretreated with vancomycin (125-250mg QID) for a minimum of four days,

followed by two liters of macrogol solution (bowel lavage) one day prior to FMT. The thawed fecal suspension is slowly infused through a duodenal tube, or via colonoscopy in selected patients.

Follow-up

Routine follow-up of patients consists of a standardized questionnaire three weeks post-FMT filled out by their local, treating physician and a telephonic interview performed by a member of the NDFB working group two months post-FMT. For this study an additional telephonic interview was performed between in January 2019, five to 33 months post-FMT. In addition, treating physicians were asked to contact the NDFB in case of any adverse events or treatment failures. Success of FMT was defined as resolution of CDI symptoms without relapse of CDI within two months. A relapse of CDI was defined as the development of diarrhea for at least two consecutive days within two months following FMT, either in combination with positive free feces toxin test or PCR (proven relapse), or clinical suspicion for CDI (probable). A CDI episode occurring at a later timepoint than two months post-FMT was regarded as a new CDI episode, as proposed by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) *C.difficile* treatment guideline [27]. The development of gastro-intestinal and other adverse events was also assessed, including nausea, vomiting, burping, abdominal pain, diarrhea not caused by rCDI, obstipation, hospital admittance, antibiotic use, and we included an open field for other complaints. In addition, participants were asked to evaluate their defecation pattern post-FMT compared to pre-FMT (improved, similar or deteriorated).

Stool samples of patients were collected before and approximately three weeks after FMT. Stool samples were preserved until use at -80°C. Patients provided informed consent for collection of stool samples and outcome data of FMT for research purposes, which was approved by the Medical Ethics Committee at the Leiden University Medical Center (P15.145).

Blastocystis sp. diagnostics and typing

Stool samples of the donors were routinely screened for *Blastocystis sp.* presence by direct microscopy of the feces and Ridley-Allen sedimentation method [28]. This

screening was performed on fresh donor stool (<2 hours after defecation). With the introduction of a specific *Blastocystis* PCR at our department in 2018, two donors were identified with negative microscopy but positive PCR for *Blastocystis* sp. In retrospect all donated fecal samples used to treat patients were tested for the presence of *Blastocystis* sp. with a specific PCR targeting approximately 360 bp of the small subunit ribosomal RNA gene (see Supplementary material). Positive samples were subtyped using sequence analysis as described previously **[29]**. Furthermore, 16 available pre- and post-FMT fecal samples of the patients treated by these two respective donors were tested with *Blastocystis* sp. PCR and when positive subsequently subtyped. *Blastocystis* sp. PCR positive patients and donors were regarded as *Blastocystis* sp. colonized

Statistics

Statistical analysis was performed using SPSS 23.0 statistical software. To test for differences between the prevalence rate of relapses and gastrointestinal symptoms of *Blastocystis* sp. positive versus negative donors and patients a Chi-square test or Fischer exact in cases of n < 5, was performed. An odds ratio (OR) was calculated using logistic regression and presented with a 95% confidence interval **[95% CI]**. For ordinal data a linear-by-linear association test was used. In addition, a Kaplan-Meier curve and log-rank test to compare CDI free survival between patients receiving *Blastocystis* sp. positive or negative donor feces was performed. For statistical comparisons, a p-value below 0.05 was considered statically significant.

Results

Blastocystis sp. positive donors

In the period between May 2016 and December 2018, 110 patients were treated with 113 FMTs, using fecal suspensions of 10 donors. In two out of 10 donors, *Blastocystis* sp. testing revealed a negative stool microcopy but in retrospect a positive PCR, with Cycle quantification (Cq) value's ranging from 18.95 to 25.13 (Table 1). Subtype analysis revealed one donor with *Blastocystis* subtype (ST) 1, and the other donor with ST3. The *Blastocystis* ST1 donor carried the *Blastocystis* for at least three donating months, and the second donor carried the *Blastocystis* ST3 for at least nine donating months.

Table 1. Details of donor to patient transfer of *Blastocystis* ST1 and ST3 by FMT

Donors				Recipients pre-FMT		Recipients post-FMT				
Donor ID	Subtype of *Blastocystis*	*Blastocystis* Cq-value	Feces collection (days pre-FMT)	Patient ID	*Blastocystis* status pre-FMT	Feces collection (days post-FMT)	*Blastocystis* status post-FMT	*Blastocystis* Cq-value	Subtype of *Blastocysti*	Colonization with *Blastocystis* due to FMT
A	ST1	25.13	119	1	neg	21	neg	n/a	n/a	no
A	ST1	23.57	199	2	neg	21	pos	25.05	ST1	yes
B	ST3	24.19	43	3	neg	20	pos	22.28	ST3	yes
B	ST3	20.16	34	4	neg	5	neg	n/a	n/a	no
B	n/aa	n/a	66	5	neg	18	pos	22.57	ST3	yes
B	ST3	19.51	64	6	neg	53	pos	27.64	ST3	yes
B	ST3	18.95	119	7	neg	15	pos	27.77	ST3	yes
B	ST3	20.94	124	8	neg	20	neg	n/a	n/a	no
B	ST3	19.81	140	9	neg	48	pos	25.78	ST3	yes
B	ST3	23.21	152	10	neg	20	neg	n/a	n/a	no
B	ST3	21.11	255	11	neg	31	neg	n/a	n/a	no
B	ST3	21.68	360	12	neg	29	neg	n/a	n/a	no
B	ST3	21.68	376	13	neg	23	neg	n/a	n/a	no
B	ST3	19.96	385	14	neg	20	pos	23.86	ST3	yes
B	n/ab	n/a	509	15	neg	20	neg	n/a	n/a	no
B	ST3	20.29	521	16	neg	27	pos	19.56	ST3	yes

Abbreviations: Cq: cycle quantification, FMT: fecal microbiota transplantation, ID: patient identification, n/a: not available or not applicable, Neg: negative, Pos: positive, ST: subtypes.

a Transplanted donor feces not available, samples 6 days prior and 2 days post-FMT positive with Blastocystis ST3.

b Transplanted donor feces not available, samples 30 days prior and 3 days post-FMT positive with Blastocystis ST3.

Patients treated with *Blastocystis* sp. containing FMT suspensions

Donor feces suspensions of *Blastocystis* sp. positive donors were used for rCDI treatment of 31 patients; four patients were treated with donor feces containing *Blastocystis* ST1, 27 with *Blastocystis* ST3. From 16 of 31 patients, stool samples pre-FMT and post-FMT were available. All fecal samples of the patients prior to FMT tested *Blastocystis* sp. negative *(Table 1)*. With a median of 20.5 days (5-53 days) post-FMT, 8 of 16 (50 %) patients developed intestinal colonization with *Blastocystis*; 7 of 14 with ST3 and 1 of 2 with ST1 *(Table 1)*. Patient DNA sequences of part of the *Blastocystis* small subunit rRNA region were 100 % identical to the sequences of their respective donors.

Patient follow-up rCDI treatment

Of the 113 FMT's performed in 110 patients to cure rCDI, 31 FMTs were performed with feces from the *Blastocystis* sp. positive donors, 82 with *Blastocystis* sp. negative donor feces. Patients treated with *Blastocystis* sp. positive donor feces had a FMT success rate (cure without relapse < 2 months) of 84 % (26/31), whereas treatment with *Blastocystis* sp. negative donor feces had a success rate of 93 % (76/82). This difference in success rate was not significant *(Table 2, Figure 1)*. Moreover, no significant difference in the number of confirmed (three versus three) and probable CDI relapses (two versus three) was found (OR 1.5, 95 % CI [0.14, 16.54], p-value 1). Of a total of 11 relapses of CDI, three were challenged by antibiotic treatment, whereas eight (five in *Blastocystis* positive and three in *Blastocystis* negative treated patients) developed a relapse without antibiotics as predisposing factor. The ST1 and ST3 *Blastocystis* sp. positive donor fecal suspensions were used for treatment of four, and respectively 27 rCDI patients. Treatment with feces of the *Blastocystis* sp. ST1 donor resulted in a treatment success of 75 % (1/4), whereas the ST3 donor had a success rate of 85 % (4/27) (OR 0.522, 95 % CI [0.04, 6.36], p-value = 0.525). In addition, no difference was found in relapse rate between patients with (12.5 %, 1/8) or without (0 %, 0/8) *Blastocystis* sp. colonization following FMT with a *Blastocystis* sp. containing donor suspension (OR 1.143, 95 % CI [0.88, 1.49], p-value 1).

Table 2. Follow-up of rCDI FMT treatment success of patients transferred with *Blastocystis* sp. positive versus negative donor feces.

Patients outcome	*Blastocystis* sp. positive donor feces	*Blastocystis* sp. negative donor feces	Significance (OR [95% CI], p-value)	
FMT success rate	83.9 % (26/31)	92.7 % (76/82)	OR 0.411 [0.12, 1.46]	p-value 0.159
Relapses of CDI	16.1 % (5/31)	7.3 % (6/82)	OR 2.436 [0.69, 8.65]	p-value 0.159
New CDI episode (> 2 months after FMT)	9.7 % (3/31)	7.3 % (6/82)	OR 1.357 [0.32, 5.80]	p-value 0.704
CDI event (relapse or new episode	25.8 % (8/31)	14.6 % (12/82)	OR 2.029 [0.74, 5.88]	p-value 0.165

Abbreviations: CDI, Clostridioides difficile infection, CI: confidence interval, FMT: fecal microbiota transplantation, OR: odds ratio.

Percentages and final odds ratio with 95 % confidence intervals of the FMT treatment outcome between patients treated with Blastocystis sp. positive versus negative donor feces. A chi-square test or Fischer exact test in cases of n < 5, was performed.

Nine (8.0 %, 9/113) patients experienced a new episode of CDI later than two months after FMT, with a median of four months (range 63 – 402 days) post-FMT. All new episodes could be attributed to initiation of antibiotic treatment shortly before development of CDI symptoms. The frequency of development of a new initial episode of CDI was not statistically different in patients transferred with *Blastocystis* sp. positive feces (9.7 %, 3/31) versus *Blastocystis* sp. negative (7.3 %, 6/82), Table 2, *Figure 1*. Moreover, no statistically significant difference in development of a new initial CDI episode was found between ST1 (0 %, 0/4) and ST3 (11.1 % 3/27) transferred patients (OR 0.889, 95 % CI [0.78, 1.02], p-value 1), or between patients that were demonstrable *Blastocystis* colonized post-FMT using *Blastocystis* containing donor feces (12.5 %, 1/8) versus demonstrable *Blastocystis* negative post-FMT (0 % 0/8) (OR 1.143, 95 % CI [0.88, 1.49], p-value 1).

Table 3. Potential side-effects due to newly acquired *Blastocystis* sp. infections after FMT

Side-effect	FMT with *Blastocystis* sp. negative donor (n=82)						FMT with *Blastocystis* sp. positive donor (n=31)						Blastocystis sp. colonized post-FMT (n=8) [a]					
	Week 1		Week 2+3		LTFU		Week 1		Week 2+3		LTFU		Week 1		Week 2+3		LTFU	
Nausea (% yes) [a]	11.0%	(9/69)	12.2%	(0/70)	35.0%	(7/20)	13.0%	(3/23)	3.2%	(1/23)	12.5%	(2/16)	0.0%	(0/8)	0.0%	(0/8)	0.0%	(0/3)
Abdominal pain [b] (% yes)	22.0%	(18/70)	18.3%	(15/71)	35.0%	(5/18)	34.8%	(8/23)	16.1%	(5/23)	25.0%	(3/12)	25.0%	(2/8)	12.5%	(1/8)	33.3%	(1/3)
Diarrhea [b]	32.9%	(23/70)	22.0%	(18/70)	35.0%	(7/20)	26.1%	(6/23)	26.1%	(6/23)	25.0%	(4/16)	0.0%	(0/8)	37.5%	(3/8)	33.3%	(1/3)
Defecation pattern																		
Improved	n/a		16.1%	(9/56)	17.6% [c]	(3/17)	n/a		13.6%	(3/22)	53.8% [c]	(7/13)	n/a		12.5%	(1/8)	33.3%	(1/3)
Similar	n/a		67.9%	(38/56)	58.8% [c]	(10/17)	n/a		68.2%	(15/22)	38.5% [c]	(5/13)	n/a		62.5%	(5/8)	66.7%	(2/3)
Worsened	n/a		16.1%	(9/56)	23.5% [c]	(4/17)	n/a		18.2%	(4/22)	7.7% [c]	(1/13)	n/a		25.0%	(2/8)	0.0%	(0/3)

[a] Subgroup of patients receiving Blastocystis sp. positive fecal suspensions with proven intestinal colonization of Blastocystis sp. post-FMT.

[b] Prevalence of nausea, abdominal pain or diarrhea were not significantly different between the groups as tested with chi-square or Fischer exact in cases of n < 5.

[c] Statistically significant difference in the self-evaluated defecation pattern at long-term follow-up between patients that received Blastocystis sp. positive versus Blastocystis sp. negative donor feces as tested by Chi-square linear-by-linear test, p = 0.043.

Abbreviation: FMT: fecal microbiota transplantation, LTFU: long-term follow-up (median 35 weeks, range 10-143 weeks), n/a: not applicable

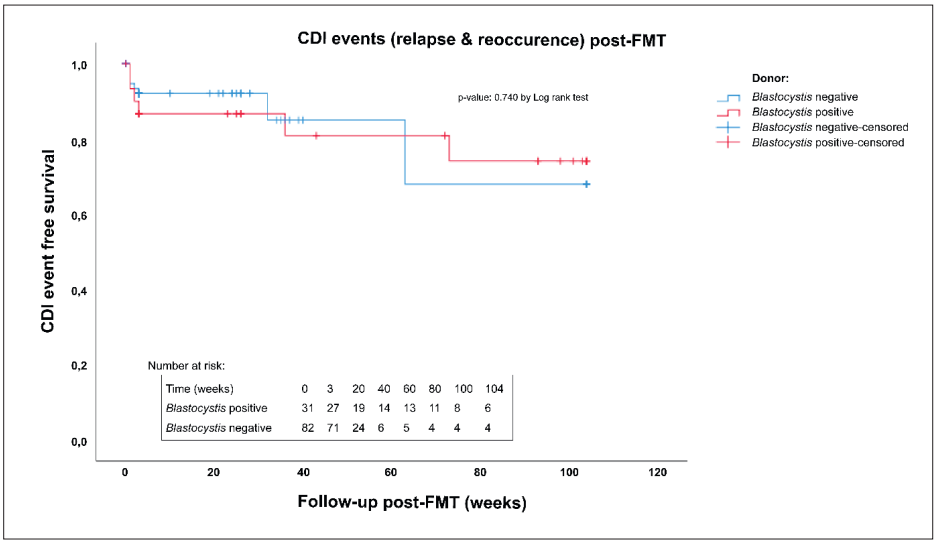

Figure 1. Kaplan Meier curve of *C. difficile* infection (CDI) event free survival in patients post-FMT treated with *Blastocystis* sp. positive versus *Blastocystis* sp. negative fecal suspensions.

CDI free survival is defined as survival without relapse (<2 months post-FMT) or new CDI infection (>2 months post-FMT) within two years (104 weeks) after FMT. Follow-up data exceeding 2 years, was censored at 104 weeks. Patients suffering from a new CDI event after 104 weeks were counted as no CDI even.

Abbreviations: CDI: *Clostridioides difficile* infection, FMT: fecal microbiota transplantation

Potential side-effects due to newly acquired *Blastocystis* sp. colonization following FMT

Compared to patients treated with *Blastocystis* sp. negative donor feces, patients treated with *Blastocystis* sp. positive donor feces did not report significantly more bowel complaints (nausea, abdominal pain or diarrhea) after one week, three weeks or at long term follow up (median 35 weeks, range 10 – 143 weeks) (Table 3). Moreover, no difference in side-effects was observed in the subgroup of patients with demonstrable *Blastocystis sp.* colonization after FMT. Interestingly, a significant difference towards an improvement of the self-evaluated defecation pattern was observed at long-term follow-up in patients receiving *Blastocystis* sp. positive donor feces (Table 3).

Discussion

Healthy stool donors colonized with *Blastocystis* sp. are usually excluded from FMT donorship [21-26], though the enteropathogenicity of *Blastocystis* sp. remains debatable [7]. Through a combination of PCR and subtyping techniques of donor, patient pre-FMT and post-FMT fecal samples, the first human to human transmission by FMT of *Blastocystis* sp. ST1 and ST3 was described. This transmission did not influence the success rate of the FMT to treat rCDI. More importantly, it did not result in gastrointestinal symptomatology of the recipients.

Symptoms attributed to *Blastocystis* sp. infection described in anecdotal case reports, series and retrospective cohorts include nausea, anorexia, abdominal pain, flatulence and acute or chronic diarrhea [8]. The high prevalence of *Blastocystis* sp. colonization in healthy individuals suggests *Blastocystis* sp. does not harm most hosts. As *Blastocystis* consists of 17 subtypes, initially the idea was raised that subtype correlated with pathogenicity [30]. Numerous, globally performed studies comparing the subtypes of *Blastocystis* could not confirm such a consistent correlation and could not explain the pathogenicity in some patients [30]. Currently, it is mostly acknowledged that *Blastocystis* sp. may colonize many hosts, but the infection's potential depends on the interplay between the virulence of the parasite, number of infecting parasites present, duration of infection (acute versus chronic) and host factors like genetics, immune competence or gut microbiota composition [3, 4, 20, 30, 31]. The two identified subtypes in this study, ST1 and ST3, are the most commonly found subtypes in Europe and the Netherlands [3]. In a Dutch study in which the stool samples of 442 patients were evaluated by routine parasitological examination, 107 (24 %) stool samples contained *Blastocystis* sp., of which 40 % *Blastocystis* ST3 and 21% *Blastocystis* ST1 [3]. The sustained colonization with *Blastocystis* ST1 and ST3 observed in 50 % (median 20.5 days) of *Blastocystis* transferred patients in this study, did not result in gastro-intestinal symptomatology, as determined by patient follow-up questionnaires. In contrast, these *Blastocystis* sp. transferred patients evaluated their defecation pattern significantly better post-FMT compared to patients receiving *Blastocystis* sp. negative donor feces.

Unfortunately, a human challenge model to study the presumed enteropathogenicity of *Blastocystis* sp. has not been described [7]. In our study, the transfer of

Blastocystis sp. was accompanied by a healthy donor microbiota. This may not reflect the effects of *Blastocystis* sp. transfer from individuals with intestinal complaints or a disturbed microbiota to individuals with a healthy microbiota. Interestingly, *Blastocystis* sp. may not be able to maintain itself in a dysbiotic rCDI microbiota, since we found that none of the rCDI patients carried *Blastocystis* sp. pre-FMT. Low *Blastocystis* sp. colonization rates in diseased individuals were previously also reported in patients with active IBD or hepatic encephalopathy **[4, 13, 14, 32]**. These diseased individuals and rCDI patients have a perturbed gut microbiota in common. Whether the association between a perturbed microbiota and low *Blastocystis* sp. colonization is a result from an absence of *Blastocystis* sp., or from the inability of *Blastocystis* to colonize and sustain in a dysbiotic gut microbiota composition is an interesting question which merits further research.

In this study the importance of performing appropriate *Blastocystis* sp. diagnostics is shown. The NDFB used microscopy on unfixed material, and Ridley-Allen sedimentation to detect *Blastocystis* sp., in contrast to the more superior techniques using microscopy on two sodium acetate formalin (SAF) fixated stool samples or molecular detection of a single stool sample **[3]**. *Blastocystis* sp. colonization of the donors or patients was, therefore, defined by positive PCR, irrespective of microscopic findings. Post-FMT stool samples with a positive *Blastocystis* sp. PCR were taken more than two weeks post-FMT. Together with the relative low Cq values (high load) found in these rCDI patients post-FMT suggests actual *Blastocystis* colonization instead of *Blastocystis* passage after FMT.

There is no consensus among FMT centers and stool banks about *Blastocystis* sp. screening of donors, though published guidelines still recommend screening, especially for immunocompromised patients **[24]**. Many centers do not screen for *Blastocystis* sp., and according to a recent systemic review only 14.5 % of 168 studies reported specific *Blastocystis* sp. screening **[33]**. In addition, the method of screening for ova and parasites was often not stated **[21-26]**. Consequently, we assume that a substantial number of patients has received FMT treatment for rCDI or other diseases in experimental setting, with co-transplantation with *Blastocystis* sp.

Our study is the first study that indicates that *Blastocystis* sp. transmission does not result in gastrointestinal symptoms of recipients. In the setting of rCDI, transmission

of *Blastocystis* ST1 and ST3 via FMT did not result in a significant decrease in efficacy of FMT, although there was a non-significant trend towards an increased rate of CDI events (both relapses and new episodes) in patients treated with *Blastocystis* sp. positive donors (8/31) versus *Blastocystis* sp. negative donors (12/82). Interestingly, this contrasts with expected outcomes as one could have extrapolated from recent metagenomic studies, in which *Blastocystis* sp. is correlated with a more diverse and healthy microbiota, a general prerequisite of a good donor **[4, 15-20]**. In a large cohort of 1106 healthy Flemish individuals, *Blastocystis* sp. carriership was associated with higher microbial diversity, richness and composition. Tito et al, found that the most common subtypes in Europe, ST1, ST2, ST3 and ST4, were all associated with a higher diversity, though ST1 and ST3 (which were identified in our study) had a lower diversity increase than ST2 and ST4 **[4]**. For FMT treatment of rCDI, super donors have not been detected **[34, 35]** and all donors display a high cure rate of around 85 % **[21]**. The role of super-donors, could play a more significant role in possible future FMT indications other than rCDI, such as ulcerative colitis, metabolic syndrome, eradication of multi-drug resistant organisms or hepatic encephalopathy **[4, 36, 37]**.

In this study only transfer of *Blastocystis* ST1 or ST3 was studied. To assess the contribution of *Blastocystis* sp. transfer to FMT success, it is important to include microbiota data of donors and patients, other subtypes of *Blastocystis*, and longer-term follow-up as colonization is described up to 6 – 10 years **[38]**. An important limitation of this study is voluntary reporting by the treating physicians of late CDI relapses (after three weeks) or new CDI episodes (after two months) to the NDFB. However, physicians had a low threshold to contact the NDFB, since an excellent relationship was developed during the entire process of FMT request and treatment of the patient.

In conclusion, to the best of our knowledge we demonstrate the first transmission of *Blastocystis* ST1 and ST3 from donor to recipient via FMT without development of gastrointestinal symptoms. This study is an important step towards a possible exempt of *Blastocystis* sp. (ST1 and ST3) as donor exclusion criterion in FMT.

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Potential conflicts of interest

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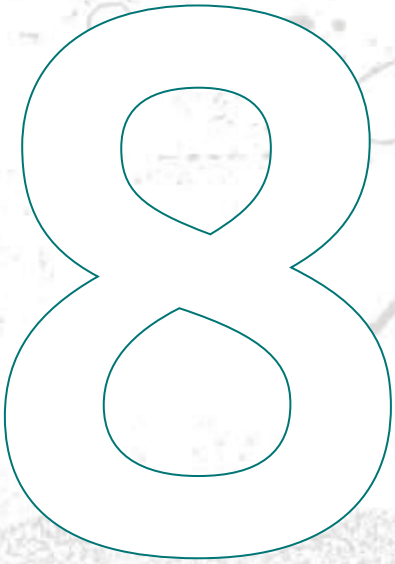

Chapter

Fecal microbiota transfer for multidrug resistant Gram-negatives; a clinical success combined with microbiological failure

Open Forum Infectious Diseases, 2017

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Chapter 8. Fecal microbiota transfer for multidrug resistant Gram-negatives; a clinical success combined with microbiological failure

Abstract

Combined fecal microbiota transfer (FMT) and antibiotic treatment prevented recurrences of urinary tract infections with multidrug resistant (MDR) *P. aeruginosa*, but failed to eradicate intestinal colonization with MDR *E. coli*.

Based on microbiota analysis, failure was not associated with distinct diminished microbiota diversity.

Introduction

Multidrug resistance (MDR) of *Enterobacteriaceae* is an increasing worldwide problem that challenges the treatment of common bacterial infections. MDR has been declared one of the greatest challenges to global public health today, and innovative strategies for decolonization of MDR bacteria are urgently needed to reduce the use of reserve antibiotics and prevent transmission [1]. A few reports mention success with fecal microbiota transfer (FMT) to eliminate extended-spectrum β-lactamase (ESBL) producing *Enterobacteriaceae*. Failures have not been reported. We present a 34-year old patient on peritoneal dialysis, treated with FMT to eradicate a Verona Integron-encoded Metallo-β-lactamase (VIM)-positive *Pseudomonas aeruginosa* causing recurrent urinary tract infections, which hampered planned kidney-pancreas transplantation. Microbiome analysis was performed prior to and after infusion of fecal microbiota.

Case description

A 34-year old male with type 1 diabetes mellitus was referred to our tertiary hospital because of diabetic nephropathy. Screening for combined kidney pancreas transplant started. Two months after starting hemodialysis, he was admitted because of bacteremia and catheter related thrombophlebitis of the brachiocephalic vein by

Staphylococcus aureus, which was treated with flucloxacillin for 6 weeks. Because the extensive thrombosis prohibited shunt or catheter placement, he was converted to peritoneal dialysis (PD). During admission, a transurethral catheter was placed because of neurogenic bladder dysfunction. Shortly after discharge he returned to our hospital with a febrile catheter-related urinary tract infection (UTI) and was treated empirically with ceftazidim. Urinary cultures were positive with a bla_{VIM} carbapenemase producing *P. aeruginosa*, resistant to carbapenems, cephalosporins, quinolones, aminoglycosides and fosfomycin, only susceptible to colistin with a MIC of 4mg/L. The same *P. aeruginosa* was isolated from a rectal swab and the PD-catheter exit site. The patient received colistin intravenously (IV) for 2 weeks and the urinary and PD catheter were replaced. In the following months, the patient suffered from recurrent febrile UTIs due to the MDR *P. aeruginosa* (details on antibiotic use shown in *Figure 1*). Because of the high likelihood of recurrence of UTI caused by this MDR organism for which the only antibiotic was nephrotoxic, kidney transplantation was considered contraindicated and the patient was removed from the waiting list. During colistin treatment of the third episode, a plan for decolonization was developed. The transurethral catheter was removed and intermittent catheterization with twice weekly prophylactic intravesical high dose gentamicin instilments was started. Repeated negative cultures of urine, PD catheter-skin interface, skin, ears and throat excluded chronic prostatitis or colonization at other sites than the gut. No oral selective digestive decontamination was given. After consultation with our ethics committee, informed consent was obtained from the patient for treatment with fecal microbiota. Six weeks after the last IV course of colistin, the infusion of FMT was performed.

Material and methods

Donor feces infusion was performed using the support of the National Donor Feces Bank (http://www.ndfb.nl/) according to the FECAL trial protocol with minor modifications **[2]**. In summary, donor feces was obtained from an unrelated healthy volunteer. Donor serum and feces were extensively screened for fecal and blood transmitted diseases including MDR bacteria. 75 gram of feces was homogenized with saline, and sieved (300µm mesh) to remove undigested food fragments. Within 8 hours after defecation of the donor, 300ml fecal suspension was infused in the duodenum of the patient through a nasoduodenal tube, after full colon lavage. Stool samples were

collected prior to infusion, after 1 week, 2 weeks, 1 month, 2 months and 3 months and screened for MDR presence using selective enrichment media, as described previously [3]. A portion of the feces was stored within 4 hours after delivery at -80°C for microbiome research. To assess the relatedness of bacterial strains, Amplified Fragment Length Polymorphism (AFLP) technique was performed as described previously [4].

Microbiota analysis

Bacterial DNA was isolated from the fecal samples using the ZR Fecal DNA MiniPrep kit (Zymo Research). Library preparation and amplification of the V4 hyper variable region 16S rRNA gene was performed using NEXTflex 16S V4 Ampliconseq kit v2.0. High-throughput sequencing was executed at ServiceXS (Leiden, the Netherlands) on the Illumina Hiseq 2500 platform (Illumina, San Diego, USA) in rapid run mode paired-end 250 base pairs read length. Raw sequences were processed and analysed using the open-source bioinformatics pipeline QIIME 1.9.1 (http://qiime.org/), the Operational Taxonomic Unit were picked using the open-reference protocol. Subsequently, microbiota profiles were reported at phylum level and visualized using the visualization tool Krona [5].

Results

No adverse event occurred during or after the infusion of microbiota, other than loose stools for 3 days. The stool culture taken prior to FMT was negative for the MDR *P. aeruginosa*, but did contain an ESBL producing *Escherichia coli*, susceptible to carbapenems, gentamicin, piperacillin/tazobactam and colistin. Subsequently 5 stool cultures up to 3 months of follow up remained negative for *P. aeruginosa*, however persisted in containing the ESBL producing *E. coli*. The *E. coli* post-FMT was identical to the *E. coli* found prior FMT, using AFLP. No infectious complications caused by *P. aeruginosa* were noted during 18 months of follow up. However, the patient was treated once with trimethoprim-sulfamethoxazole for cystitis caused by an ESBL positive *E. coli* 8 months after FMT. Unfortunately, this strain was not available for AFLP analysis.

16S analysis of the patient's stool 19 and 1 days prior FMT revealed a diverse microbiota composition, i.e., high Shannon diversity index of 7.8 and 8.1 respectively.

No significant changes in microbiota diversity of the recipient were observed following the FMT *(Figure 1)*. At phyla level a high similarity of donor and recipient microbiota was observed with respect to the *Firmicutes* and *Bacteroidetes* as the expected main phyla of the microbiota *(Figure 1)*.

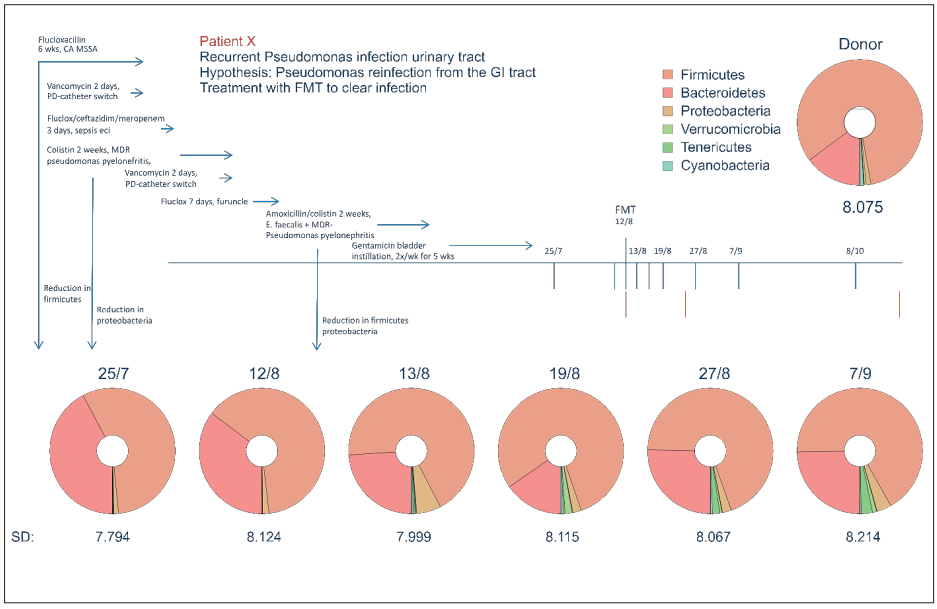

Figure 1. Timeline of recurrent infections, antibiotic use, and microbiota diversity prior to and after fecal microbiota transfer.

CA MSSA: catheter related bacteremia with methicillin sensitive *Staphylococcus Areus*.
MDR: multidrug resistant. PD: peritoneal dialysis. FMT: fecal microbiotica transfer.

Discussion

A 34-year old patient on peritoneal dialysis and recurrent urinary tract infections with a VIM-positive *P. aeruginosa* was treated with infusion of fecal microbiota to eradicate *P. aeruginosa* from the intestinal tract. A clinical success was observed, since at a follow-up period of 18 months no recurrent infections by *P. aeruginosa* were diagnosed. FMT may have contributed to clinical success but it cannot be excluded that MDR *P. aeruginosa* was already eradicated from the gut before FMT, as the *P. aeruginosa* could not be cultured the day before FMT.

A remarkable observation is the persistence of an ESBL positive *E. coli* after FMT. The *E. coli* was presumably acquired after eradication treatment for *P. aeruginosa*, since it had not been detected in earlier cultures. It is possible that the incomplete eradication of the MDR *E. coli* is the result of coexistence of donor and patient *E. coli* strains after FMT. A recent study showed this coexistence of donor and recipient strains, which persisted for at least 3 months after FMT for treatment of patients with metabolic syndrome **[6]**. This suggests that novel strains, acquired via FMT, can colonize the gut without replacing the indigenous strain population of the recipient.

In contrast to the diminished microbiota of recurrent CDI patients, our patient had an intact microbiota diversity and composition at phylum level prior to FMT. Previous antibiotic treatment *(Figure 1)* had not resulted in a distinct disturbance of the intestinal flora. Only minor changes of the microbiota composition were observed after FMT with a slight increase of cyanobacteria and tenericutes. We suggest that diminished diversity appears not to play a role in MDR carriership as opposed to recurrent CDI **[7]**. Therefore, one might question the efficacy of fecal transplantation in patients with a normal microbiota diversity. The disturbed microbiota and its recovery after FMT might explain the positive results of MDR eradication in patients with recurrent CDI **[8, 9]**. Interestingly, a recent paper showed that infusion of fecal microbiota in patients with recurrent CDI decreased the number and diversity of antimicrobial resistance genes, particularly by restoring dysbiosis and reducing the number of *Proteobacteria* **[10]**. Furthermore, beneficial effect of microbiota transfer has been shown in mice colonized with vancomycin resistant *Enterococcus* (VRE) **[11]**. Clearly, more research on FMT for eradication of colonization of different MDR bacterial species is required.

A total of only eight case reports have been published, showing FMT resulted in intestinal decolonization of ESBL- and carbapenemase-producing Enterobacteriaceae, VRE, or methicillin-resistant *Staphylococcus aureus* **[12-15]**. Unfortunately, no information has been provided on microbiota composition before and after transplantation. Five trials are currently underway regarding the use of FMT for MDR bacterial decolonization which should provide more insight on the role of the microbiota on colonisation with specific microorganisms **[12]**.

A limitation of our analysis is that the microbiota was determined by 16S analysis. Although very useful in bacterial taxonomic classification, it lacks the required resolution to track transmission of bacterial strains in the microbiota using single-nucleotide variants in metagenomes [6]. Therefore, it was not possible to compare the composition of the microbiota at strain level, allowing a comparison between the donor and patient *P. aeruginosa* strains. However, no VIM-gene was detected by PCR on DNA from three feces samples after FMT.

In conclusion, combined FMT and antibiotic treatment prevented recurrence of UTI with MDR *P. aeruginosa*. Intestinal colonization with ESBL producing *E. coli* persisted in the presence of a microbiota with intact diversity, suggesting that eradication of *E. coli* requires perhaps other specific strain(s) of microbes. More detailed analysis such as metagenomics, could identify specific strains that add to decolonization and should be applied in current studies on FMT for intestinal eradication of different MDR bacterial species.

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Chapter 9

Discussion

Summary of main findings
General discussion
Nederlandse samenvatting
List of publications
Curriculum Vitae
Dankwoord

Chapter 9. Discussion

Summary of main findings

Part I: New insights in the epidemiology of *Clostridioides difficile* and multidrug resistant Gram negatives.

Infections with *C. difficile* have long been considered as a nosocomial acquired diarrheal disease, transmitted primarily from symptomatic patients. However, transmission of *C. difficile* spores to the hospital environment, health care workers and other patients is not only accommodated by infected patients, also asymptomatically colonized individuals shed these microorganisms [1]. Recognition of asymptomatically colonized patients is essential in reducing nosocomial transmission. In **Chapter 2** the performance of several diagnostic *C. difficile* tests in comparison to toxigenic culture as gold standard was evaluated in asymptomatically colonized patients at admission to three large hospitals in the Netherlands. In this study, 5.1% of the patients attending a tertiary-care hospital were positive with *C. difficile*, and 3.1% contained toxigenic *C. difficile*. In a setting of low endemicity of asymptomatically colonized individuals, all three assays (an enzyme-linked fluorescent assay (ELFA) targeting glutamate dehydrogenase, and two molecular tests targeting the toxin(s) of *C. difficile*; a commercial *artus* PCR which targets *TcdA* and *TcdB*, and an in-house PCR only targeting *TcdB*) can be applied as a first screening test to detect the presence of (toxin producing) *C. difficile*, as they display a very high negative predictive value. Similar to the diagnosis of patients with symptomatic *C. difficile* infection (CDI), the positive predictive values of the tests in a low endemicity setting were suboptimal. Discrepancy analysis demonstrated that the majority of the small number of false positive results could not be confirmed upon retesting. This illustrates that in a low prevalence setting a positive GDH or PCR result is not automatically based on an increased sensitivity of these assays as compared to the toxigenic culture as gold standard.

Nosocomial transmission by asymptomatic carriers is also recognized for MDRO. Prevention of this transmission in hospitals is pursued by screening selected patient groups for carriership of MDRO. The approach varies per country and per micro-

organism, as illustrated by the variety of national guidelines. In 2015, a novel plasmid mediated resistance mechanism of colistin, *mcr-1* was discovered in animals and humans in China [2]. Currently, ten *mcr* genes types (*mcr-1* to *mcr-*10) have been detected in Enterobacterales isolates [3]. Epidemiological data on the prevalence of faecal carriage of *mcr-1* in healthy individuals were not available shortly after the discovery of this novel resistance plasmid. To assess the risk of *mcr* introduction from asymptomatic carriers into our academic tertiary care hospital, the prevalence of *mcr* in faecal samples obtained from patients attending our hospital was investigated in **Chapter 3.** Two of the 576 (0.35 %) patients tested positive for *mcr-1*, whereas no *mcr-2* was found. This suggests that *mcr* spread from the community into the hospital environment was low in the Netherlands but could not be excluded. The finding of a phenotypically colistin susceptible, *mcr-1* plasmid containing *E. coli* underlined the importance of phenotypical confirmation after molecular screening.

In contrast to healthy young individuals in the community, nursing home residents have multiple risk factors for colonization and infection with *C. difficile* and MDRO [4-10], and are considered a reservoir for transmission [11-14]. Frequent contact between residents due to communal living, increased frequency of healthcare contact and presence of factors that facilitate MDRO spread such as incontinence provide additional opportunities for transmission. The data presented in **Chapter 4** show that a high abundance of *C. difficile* and MDRO risk factors was present in Irish and Dutch nursing home residents. Surprisingly, this did not result in a high prevalence of MDRO's; 9 % and 11% Extended Spectrum Beta Lactamase (ESBL)-producing *E. coli* in Ireland and the Netherlands respectively, and 0 % carbapenemase producing Enterobacterales (CPE), vancomycin resistant Enterococci (VRE) or *C. difficile* in both countries. Using core-genome multi locus sequence typing (cgMLST) small-scale spread of MDROs between residents of the same ward in the Netherlands was demonstrated. However, cross-transmission of MDRO's between three different wards in Ireland was observed by whole genome sequencing. The differences between Ireland and The Netherlands may reflect differences in nursing home infrastructure, specifically communal areas and multi-bedded resident rooms in the Irish nursing home, which were not present in the Netherlands.

In conclusion, though asymptomatic colonization of MDRO and *C. difficile* can become a nidus for nosocomial transmission, its prevalence is still low in the Netherlands.

Part II: The initiation of the Netherlands Donor Feces Bank to facilitate quality assured faecal microbiota transplantation

Faecal microbiota transplantation (FMT) has become an established treatment for rCDI and is extensively studied as new treatment option for many other indications. As result, stool banks focussing on standardisation, safety, quality assurance and cost effectivity become increasingly important [15-17]. Stool banks provide ready-to-use faecal suspensions to hospitals for treatment of patients. **Chapter 5** describes the establishment of the Netherlands Donor Feces Bank (NDFB). It addresses the difficulties encountered with donor recruitment and screening, preparation of the faecal suspension and transfer of the faecal microbiota suspension. It also provides treatment data and follow-up of patients treated with donor faeces of the NDFB. In comparison with the experiences of others stool banks, the NDFB has a high cure rate of rCDI at two months of nearly 90 % and a sustained cure rate of over 70 % after a mean follow-up of 42 weeks (**Chapter 6**). This high success rate is most likely achieved by the efforts of our multidisciplinary FMT-expert panel. This expert panel discusses the indication for FMT for patients for whom an FMT is requested and provides advice during treatment and follow-up of the patients. This strategy results in efficacious, appropriate and safe use of FMT for treatment of rCDI.

Healthy stool donors colonized with *Blastocystis* sp. are usually excluded from FMT donorship [21-26], resulting in considerable exclusion of donors (30-50 %). It is questionable whether this is justified as the entero-pathogenicity of *Blastocystis* sp. is debatable. The presumed pathogenicity is based on anecdotal case reports and retrospective reviews and a human challenge model has not been applied [7]. Recent literature shows a lower prevalence of intestinal carriage of *Blastocystis* sp. in patients with disorders associated with a reduced diversity of the gut microbiota, such as inflammatory bowel disease or hepatic encephalopathy [18-21]. Metagenomic studies reveal furthermore that *Blastocystis* sp. correlates with a more diverse and healthier microbiota [18, 22-27]. Through a combination of PCR and subtyping techniques of faecal samples of donors and patients (pre-FMT and post-FMT), the first human to human transmission by FMT of *Blastocystis* sp. ST1 and ST3 is described in **Chapter 7**. This transmission did not influence the success rate of the FMT to treat rCDI. More importantly, it did not result in gastrointestinal symptomatology of the recipients. This

study is an important step towards a possible exempt of *Blastocystis* sp. (ST1 and ST3) as donor exclusion criterion in FMT.

In **Chapter 8** the potential of FMT for eradication of MDRO was explored in a patient suffering of recurrent urinary tract infections with a carbapenemase producing *Pseudomonas aeruginosa* hampering planned kidney-pancreas transplantation. Antibiotic pre-treatment subsequently followed by FMT prevented recurrence of a urinary tract infection with this Verona integron-encoded metallo-β-lactamase (VIM) positive *P. aeruginosa* by eradication of intestinal colonization. Although the treatment was a clinical success, a partial microbiological failure was observed as intestinal colonization with an ESBL-producing *Escherichia coli* persisted. In contrast to the diminished microbiota of rCDI patients, microbiota analysis showed an intact microbiota diversity and composition at phylum level before FMT. This suggests co-colonization rather than replacement of indigenous strains and eradication of this MDRO *E. coli* requires perhaps other microbiota interventions.

With the increasing number of reports pointing towards potential beneficial effects of FMT in patients with a variety of gastrointestinal and extra-intestinal disorders, a growing demand of FMT can be expected in the near future. Initially, experimental studies will have to be performed in a controlled setting, both *in-vitro* and *in-vivo*. Once proven effective, a standardised screening and manufacturing procedure, quality control and careful and long-term monitoring of outcomes and adverse events requires stool banks and registries. The experience of this thesis and the NDFB may help the establishment, utilization, standardization and maturation of stool banks throughout Europe, and FMT as therapy.

General discussion

Targeting the pathogen alone is often not sufficient for diagnosis and treatment of CDI

Diagnosis of CDI

To prevent inappropriate use of FMT and increase the clinical benefit and cost-effectiveness, evaluation of FMT requests by a multidisciplinary team is extremely important. The NDFB FMT-expert team rejects a quarter of FMT requests, which is in the majority of cases (30/47, 64%) because the diarrhoea was attributed to another cause that coincided with *C. difficile* carriership (**Chapter 6**). In the past, *C. difficile* was difficult to isolate and cultivate from other anaerobic and facultative members of the gut microbiota. Currently, many molecular tests are available to demonstrate the presence of *C. difficile* and cultivating is not routinely applied anymore. However, the new diagnostic challenge is to distinguish colonization from infection. Due to the possibility of asymptomatic colonization and diarrhoea due to another cause, presence of the bacterium in the faeces does not consequently indicate disease. ESCMID and the ECDC recommend a two-stage algorithm with a screening test with-high sensitivity (nucleic acid amplification test (NAAT) or glutamate dehydrogenase enzyme immune assay (GDH EIA)) followed by a specific assay for free toxin detection in the stool (toxin EIA) [28]. The presence of free toxins in faeces is considered as the best proof for active *C. difficile* disease [29, 30].

New developments in diagnosis of CDI

In order to simplify this diagnostic approach and minimize multiple testing, alternatives have been explored. The NAATs have gained much popularity in recent years because of their ease of use and the ability for automation and standardization. We and others have found that the cycle quantification threshold (Cq) value can be a predictor of free toxin presence as measured with toxin EIA [31-34], clinical disease [35, 36] or poor outcome [33, 37, 38]. In addition, in a study reporting both PCR, as well as a predicted toxin result based on Cq-value, all patients with CDI related complications were predicted correctly. This strategy furthermore reduced the treatment of toxin-negative patients [39], and can augment a more timely diagnosis in the more severe CDI cases or clear carrier cases (for instance: Cq value < 25 = CDI,

Cq value > 33 = carrier). It is however, still not sufficiently specific to use PCR as stand-alone tests for CDI diagnosis, with a grey area of intermediate high Cq value of 25-33 in which clinical assessment, a toxin EIA or cell cytotoxicity neutralization assay (CCNA) is necessary. Attempts to enhance the sensitivity of tests able to detect free toxin in stool have been unsuccessful so far. An example is a next generation enzyme immune assay; the ultrasensitive single-molecule array (SIMOA) technology [40]. This ultrasensitive assay is capable of separately detecting and quantifying *C. difficile* toxins A and B down to picogram-per-millilitre levels. This more sensitive way of detecting free toxin in stool, increases the sensitivity to 85.2-100 % compared to CCNA [40-43]. This highly sensitive toxin assay may however suffer from the same specificity problem (with a reported specificity of 79.3 % [42]) as the highly sensitive molecular tests, which can lead to the erroneous identification of colonized individuals as diseased [44]. Although a decreased specificity was not reported in all studies [40, 41, 43, 45]. Pollock and colleagues showed that on individual patient level both PCR as SIMOA could not distinguish a patient with CDI from asymptomatic carriage [46]. Only on population level *C. difficile* toxin concentrations measured by SIMOA but not with NAAT, were significantly higher in CDI patients than in colonized individuals [46]. This is in agreement with a study showing that the rate of asymptomatic *C. difficile* carriage was similar to the symptomatic positivity rate [47]. These results suggest that part of the PCR and SIMOA positive samples in symptomatic patients are likely due to *C. difficile* colonization, and exclusive reliance on highly sensitive tests results in overdiagnosis, overtreatment and increased health care costs.

Personalized CDI diagnostics

A complicating factor in the discrimination between infection and carriership of *C. difficile* is that only the presence of free toxins in the faeces as measured with CCNA highly correlates with clinical *C. difficile* disease [29, 48]. The CCNA test however, is only performed in reference laboratories and is too cumbersome for routine diagnostics. In contrast, the routinely applied toxin EIAs lack sufficient sensitivity with a pooled sensitivity of 83 % [28]. In addition, due to frequent testing in a low prevalence setting, the high specificity of 98-99 % can result in a low positive predictive value (69-81% when the prevalence of CDI is 5 %) [28]. How can we then differentiate between colonization and infection? A personal view on personalised diagnostics is visualised in *Figure 1*. To enhance the discrimination, disease specific biomarkers can be included

in the test-algorithm, such as host inflammatory markers, stool metabolites and microbiota analysis in addition to multiplex testing for additional pathogens and virulence characteristics of *C. difficile* (e.g. RT027) [49]. The serum presence of the chemoattractant C-C motif ligand 5 (CCL5), which is expressed by many cells and actively recruits leucocytes to inflammatory sites, was associated with CDI as compared to patients with non-CDI diarrhoea [50]. Moreover, severe CDI patients had higher serum levels of TNF-α, procalcitonin, hepatocyte growth factor, IL-6 and/or IL-8 suggesting worse inflammation [50-54], and a serum based biomarker panel could inform about CDI diagnosis, treatment response and mortality [54]. Interestingly, CCL5, which marks acute intestinal inflammation and severe CDI, seemed also to be significantly associated with an increased survival [51]. Assessment of local host responses, with faeces as proxy, may be a more sensitive disease indicator than the systemic response. Faecal levels of biomarker as TNF-α, CXCL-5 messenger (m)RNA, IL-8 mRNA and IL-8 protein, lactoferrin, calprotectin and procalcitonin at initial presentation correlated with disease severity or persistent diarrhoea [49, 55-59], and were more sensitive than clinical severity scores or organism burden in identifying patients at risk for treatment failure [60].

Diagnostics in relation to FMT indication

Measurement of the humoral immunity against *C. difficile* can play a significant role in the detection of patients who will most likely benefit of immunity enhancing anti-*C. difficile* therapy with bezlotoxumab, since a higher risk of recurrence of CDI is associated with low serum concentrations of antibodies directed against the toxins TcdA and TcdB [61-63]. High serum endogenous IgG antibodies on day 1 against *C. difficile* toxin B but not toxin A, were associated with protection with rCDI after bezlotoxumab, although the effect was limited (25% versus 35% relapse) [64]. Furthermore, biomarkers can play a significant role in the evaluation of FMT-candidates, which has proven to be difficult especially in patients with inflammatory bowel disease (**Chapter 6**). The biomarker procalcitonin can assist in differentiating infection from colonization with *C. difficile* in ulcerative colitis (UC) patients, as serum procalcitonin was significantly elevated in UC patients responding to CDI treatment, in comparison to UC patients diagnosed with a UC flare in combination with *C. difficile* colonization [65]. Moreover, it could support the identification of patients potentially benefitting of repeat FMTs, as faecal calprotectin concentrations just prior FMT were higher in rCDI patients that needed multiple FMT treatments [66]. The composition of

the microbiota can also be used to predict the likelihood for CDI recurrence. Compared to non-recurrent CDI, patients with rCDI have a diminished bacterial diversity and species richness, and significant shifts of *Escherichia/Shigella* (Enterobacterales), *Veillonella,* Streptococci, *Parabacteroides* and *Lachnospiraceae* [67, 68]. Interestingly no particular taxon seemed to be associated with the severity of CDI, likely reflecting the dominant role of host-related factors [67]. Reconstitution of a healthy microbiota after FMT both defined by a high diversity or by an alteration in abundance of specific taxa or restoration of functions (e.g. bile acid conversion, short chain fatty acid production), showed to be an excellent predictor of clinical response after FMT [69, 70]. A prediction model based on 16S analysis of faeces at day seven post-FMT (with freeze dried capsules), which included the abundances of members of the families *Lachnospiraceae, Ruminococcaceae, Bacteroidaceae, Porphyromonadaceae* and *Enterobacteriaceae* showed an accuracy of 100 % and 97 % in predicting recurrences of respectively training and test data (n=89) [69]. In addition, a higher engraftment of donor strains (50 %) was observed in the responders [69]. Alternatively, bacterial fermentation products can be measured as read-out of a successful FMT, as a combination of urinary p-cresyl sulphate and the faecal concentrations of lithocholic acid seven days post-FMT could predict FMT success with high accuracy [71].

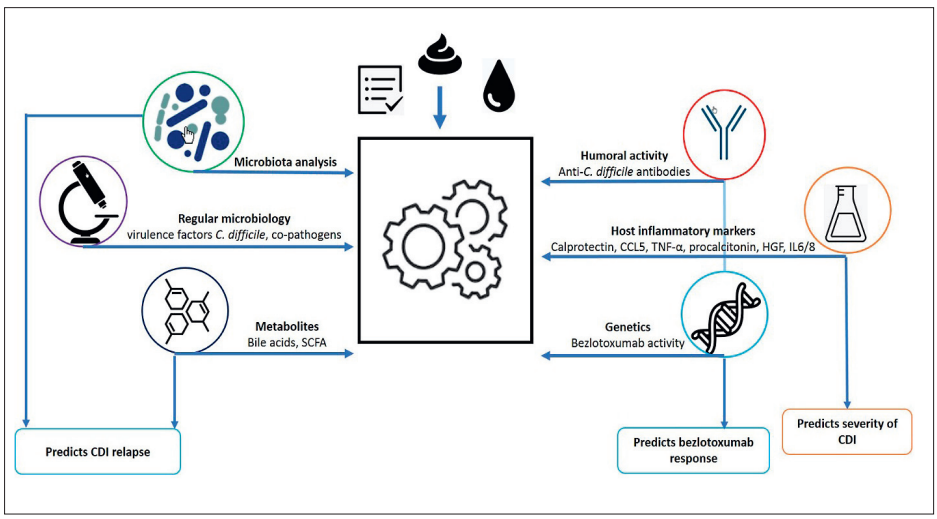

Figure 1. Personalised diagnostics for CDI

The future potential of personalised CDI diagnostics for enhanced discrimination between *C. difficile* colonisation and infection, and improved prediction of disease severity and treatment outcome.

Personalized treatment of rCDI

Personalized diagnostics can optimize treatment and subsequently improve the outcome of CDI. For a subset of patients, targeting the pathogen *C. difficile* alone is proven to be insufficient for sustained cure [72]. Depending on the above described diagnostic outcomes, the patient can be categorized as having different systems failing. Each system failing requires a different treatment approach. Therefore, I would envision the following treatment strategy *(Figure 2)* based on the assumptions mentioned below:

- √ A diverse and healthy microbiota is important for resilience against disease, not only to CDI, but also to other intestinal and extra-intestinal diseases. The aim of CDI therapy should therefore be to restore the patients' perturbed microbiota.

- √ Fidaxomicin replaces vancomycin, because of the severe impact of vancomycin on the indigenous microbiota [73]. Fidaxomicin is proven to be evenly effective in resolving CDI and preserves the microbiota, thereby resulting in fewer relapses after treatment [74-77]. In addition, although fidaxomicin is more costly than vancomycin or metronidazole, it was proven cost-effective due to averted mortality, utility loss, and costs of rehospitalisation and/or further treatments of rCDI [78]. Although fidaxomicin is superior in gain of quality-adjusted life years, the cost-effectiveness differs between studies in various countries [79, 80], and a definite conclusion is difficult to make. However, preliminary data from the new IDSA and ESCMID guidelines (2020-2021) show that fidaxomicin will probably become the first agent of choice for CDI treatment.

- √ Extended-pulsed fidaxomicin (taper therapy) is superior and more cost-effective than a regular scheme of fidaxomicin [81, 82]. However, the presumed decreased compliance of the patients to follow the more difficult treatment regime does not justify the extended-pulsed approach for a first CDI episode, but could be given for patients with a recurrence.

- √ FMT is both more effective and less costly than any other antimicrobial therapy for CDI [83-86]. Because of the unstandardized nature of this treatment and potential (low) risk for transfer of unrecognized pathogens or disease traits, treatment with anti-CDI antibiotics is preferred before FMT is administered. Consequently, FMT should be considered as treatment for the first relapse of CDI *(Figure 2)*.

- √ Bezlotoxumab is a human monoclonal antibody against *C. difficile* toxin B. Especially patients with a higher risk for rCDI (age ≥65 years, history of previous CDI, compromised immunity, IBD, renal disease, severe CDI or CDI with ribotype 027/078/244)may benefit of bezlotoxumab [87-89] to prevent relapses. When added to anti-*C. difficile* antibiotics it enhances the resilience for CDI relapses in general with ~40-50 % (relapse rate with vancomycin versus vancomycin + bezlotoxumab was 8 % vs 17 % and 16 % vs 26 %), but can be higher in particular patient groups with enhanced risk of rCDI [88, 90]. Its efficacy appears to be due to prevention rather than delayed onset, as sustained cure was observed of 69 patients cured at 12 weeks after treatment [91].

- √ The clinical relevance of the difference in absolute relapse reduction by bezlotoxumab (~9-10 %) versus fidaxomicin (~10-16 %) can be questioned. Bezlotoxumab is mainly studied in vancomycin and metronidazole treated patients, and only in small groups of patients using fidaxomicin [90, 92]. If both therapies are additive to each other remains therefore unknown although they certainly could be due to the different working mechanism [75, 77]. The combination of fidaxomicin with bezlotoxumab has been successfully applied in a few patients (n=10) with multiple rCDI for which FMT was contraindicated (personal communication prof. Maria Vehreschild and prof. Ed Kuijper).

- √ A recent exploratory genome-wide association study revealed three genetic variants located in the extended major histocompatibility complex (MHC) that were associated with a two to three fold reduction of *C. difficile* relapses in bezlotoxumab treated patients [93]. Around 40 % of patients have these genetic variants. This suggest a host-driven, immunological mechanism in response to bezlotoxumab. If these alleles are confirmed in a validation study, a human genetic analysis can be used to personalise CDI treatment.

- √ Bezlotoxumab is considered less effective in patients with multiple recurrent CDI (≥2 episodes) but prospective studies are missing [90, 92]. The LUMC Center for Infectious Diseases (LU-CID) and NDFB are therefore currently designing a randomized controlled trial to assess the efficacy of vancomycin + bezlotoxumab compared to FMT for the treatment of multiple recurrent CDI.

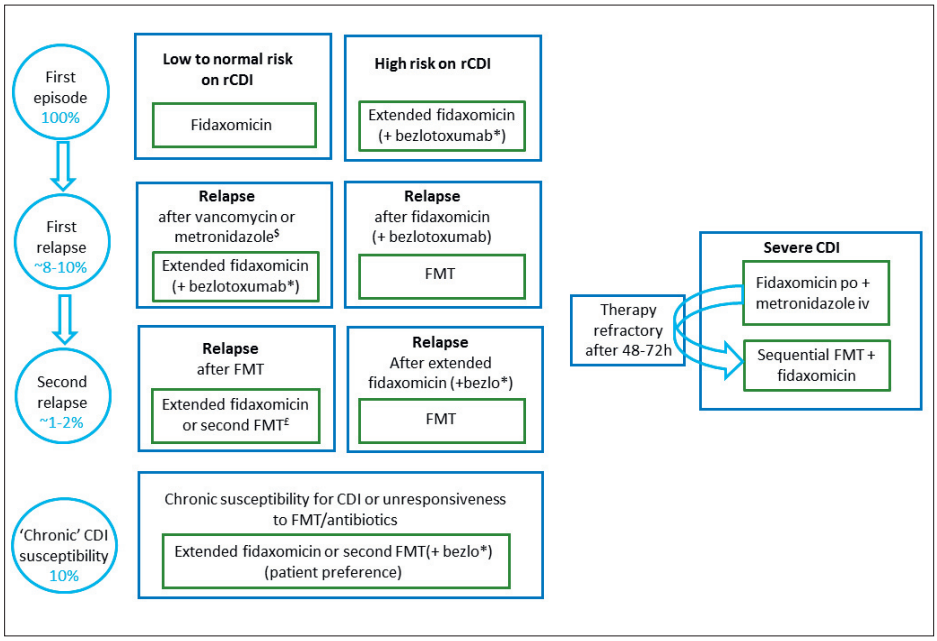

Figure 2. Personalised treatment for CDI

The future potential of personalised CDI treatment based on the in the text-mentioned assumptions. The blue circles on the left reflect the estimated percentage of patients that has the specific type of CDI (a first CDI episode is set at 100 %). The dark blue boxes give information on the indication, the green boxes inside about the proposed therapy.

* In case a genetic variant of the major histocompatibility complex (MHC) is present (~40 % of patients); this predicts a two to three-fold reduction in CDI relapses with bezlotoxumab use in comparison to patients without these genetic variants.

$ In case this personalized treatment is not followed, and metronidazole or vancomycin is given.

£ Based on shared decision making between patient and physician. Patients with 'Chronic CDI susceptibility' have suffered from multiple (recurrent) episodes in the past, but do not meet the criteria of recurrent CDI (CDI relapse within two months after prior episode) with the present episode.

Patients with high susceptibility for CDI have suffered from multiple (recurrent) episodes in the past, but do not meet the criteria of recurrent CDI (CDI relapse within two months after prior episode) with the present episode.

Mechanism of action of faecal microbiota transplantation

Mechanism of action of FMT to prevent relapses of *Clostridioides difficile* infection

The mechanism of FMT is likely multifactorial. Reinstatement of a robust and diverse, functional microbiota is an essential mechanism for resilience against CDI relapses [94]. FMT was found to restore both short chain fatty acid levels and bile acid metabolism (bile salt hydroxylation (BSH) as well as 7-α-dehydroxylation) [95-98]. In a mouse model, the bile acid converting *Clostridium scindens* (7-α-dehydroxylase) bacterium was inhibitory to *C. difficile* [99]. The co-administration of other bacterial species from *Lachnospiraceae* and *Porphyromonadaceae* families enhanced the protective potency [99]. An additional effect of 7-α-dehydroxylase producing bacteria, is the secretion of tryptophan derived antibiotics; 1-acetyl-β-carboline and turbomycin A [100]. These antibiotics inhibit the cell division of *C. difficile*, and the activity of tryptophan is enhanced by secondary bile acids [100]. However, reconstitution of the bile acid converting microorganisms with synthetized bacterial communities or live biotherapeutic products (LBPs) enhance the colonisation resistance to *C. difficile* but are not sufficient for complete prevention of acquiring CDI or sustained cure of CDI. To date, no synthetic bacterial community has achieved a success comparable to CDI antibiotics or FMT though recent interim analyses of some phase 3 studies are promising. In a proof of principle phase I study with a fractionated and encapsulated bacterial spores product (SER-109 with spores from approximately 50 bacterial species), following standard of care, most patients (29/30) achieved clinical resolution [101]. However, SER-109 failed to be of additional benefit compared to standard of care in a phase II study in rCDI patients (44 % relapse versus 53 % in placebo, n=89) [102]. Recently, SERES claimed via twitter that SER-109 did met their phase III primary endpoint, showing a statistically significant 30 % absolute reduction in rCDI compared to placebo (relapse rate of 11 % versus 41 % in placebo) [103]. A remarkable finding in both studies is the high rate of relapses that occurred in the control group. This could either be explained by study inclusion of patients with a high risk on rCDI or inclusion of patients colonised with *C. difficile*. Various studies with rational selected bacterial consortia or faeces microbiota derived products (VE303 of Vedanta, Finch, Rebiotix) are underway and show promising preliminary results.

The gut microbiota does not solely consist of bacteria but does contain various other microorganisms. Recently, it was shown that the presence of *Candida albicans* in the intestinal tract reduces the efficacy of FMT [104]. In contrast, healthy donors and patients responding to FMT displayed a high relative abundance of *Saccharomyces* and *Aspergillus* [104]. A recently completed pilot study of NDFB and CMAT (Center for Microbiome Analyses and Therapeutics) analysing the mycobiome of healthy individuals and patients either infected or colonised with *C. difficile* confirms this observation. A relatively high abundance of *C. albicans* in CDI patients, and more *Saccharomyces* and *Aspergillus* in non-CDI patients and healthy donors was observed (Zwittink, unpublished observation).

In patients with severe and therapy refractory CDI, administration of FMT results in fast but sometimes temporary improvement of clinical symptoms within hours [105-107]. The temporary improvement is sufficient to deescalate the clinical status, enabling a response to repeat FMTs [106, 107]. In this short period, stable engraftment of a functional microbiota is unlikely and inhibition of intestinal inflammation is likely to play a significant role [107]. The afunctional and unbalanced patient's inflammatory response could be reshaped by yet not fully understood mechanisms and compounds, as the microbiota impacts various immune pathways that aid in recovery from CDI colitis [108, 109]. An interesting target is interleukin-33 (IL-33), an important guardian of the gut barrier during *C. difficile* colitis that prevents CDI-associated mortality via activation of group 2 innate lymphoid cells [110]. Intestinal IL-33 expression is regulated by the microbiota, and FMT was proven to rescue the antibiotic-associated depletion of IL-33 [110]. Also regulatory T-cells play a critical role in the maintenance of immune homeostasis and seem an interesting immunological target [109]. FMT was shown to control inflammation and colitis via induction of regulatory T cells [111, 112]. Regulatory T cells are activated by many different pathways and different bacteria, for instance via commensals activating IL-10 and/or TGF-β which recruit the regulatory T cells to the intestine [111-114], via bacterial polysaccharide A that results in inhibition of IL-17 and thereby an increase in regulatory T-cells [113], or via short-chain fatty acids that promote the fitness and differentiation of regulatory T-cells [115, 116]. Whether FMT-directed immunosuppression aids also in the recovery of CDI colitis requires further investigation [109].

In addition to direct amelioration of severe CDI symptoms by immunomodulation through faecal suspension, a direct impact of the FMT can also be achieved by bacteriophages [117] or production of bacteriocins such as thuricin CD or nisin by the living bacterial fraction of the faecal suspension [118-120]. In addition, the toxin expression of *C. difficile* could be suppressed by carbohydrates present in the faecal suspension [94, 121], or human donor metabolites such as alpha-defensins [122]. The toxins could be rapidly neutralized, as bile acids reversibly bind to *TcdB*, causing a 'balled up' formation of the toxin which is no longer able to bind to the host's cell surface receptors [123].

In conclusion, it is likely that not a single bacterium or bacterial community contribute to colonisation resistance and prevention of CDI relapses by FMT. Multiple microbiota communities and networks (including bacteria, viruses, eukaryotes etc.) exist that enhance resilience or protection to rCDI. In addition, by faecal microbiota transplantation a complete functional ecosystem is transplanted. The effect of FMT is the result of a complex interplay of microbiota networks, immune modulation and metabolites that not only influence the colonisation resistance to *C. difficile* but also affect host inflammation and bacterial toxin production.

Mechanism of action of FMT for eradication of multidrug resistant organisms

Intestinal colonization of MDRO, and general decolonization strategies

Most infections with ESBL producing Enterobacterales are preceded by intestinal colonization [14, 124], and prevention and eradication of these MDRO from the gut is therefore of interest. Spontaneous intestinal clearance of an ESBL containing micro-organism varies per bacterial (sub)species and per ESBL enzyme. Duration of colonisation is on average longer in patients with comorbidity; 43 % remained ESBL positive after 1 year [125]). Contrary, healthy individuals in the general population had a mean duration of ESBL colonisation of 4.2 months [126] and 33 % remained MDRO positive for > 8 months [127]. Individuals who travelled had a median duration of ESBL colonisation of 30 days, whereas only 14.3 % and 11.3 % remained colonized at 6 and 12 months after return, respectively [128]. Spontaneous clearance of certain *E. coli* sequence types (ST) appears to be more difficult, as colonization of *E. coli* ST131 is

associated with a longer duration of carriage in a long-term care facility residents, with a half-life of 13 months versus 2- to 3- months for other STs [129]. Presence of the MDRO in the intestinal tract below the limit of detection in faeces can sometimes complicate study outcomes or interpretation. This phenomena is well known for detection of vancomycin resistant enterococci (VRE), as on average four to five rectal swabs, collected on separate days, are needed to detect >90 %-95 % of new VRE carriers [130, 131]. Especially in the first stages of colonisation, just after a transmission event, VRE detection using rectal or perianal swab can be less sensitive than faeces samples [132, 133]. Difficulties with detection of MDRO in the intestinal tract due to low levels is also observed for Gram negatives [134, 135]. A RCT that studied the decolonisation effects of a combination of colistin and neomycin versus placebo observed a significantly lower rectal carriage of ESBL in the non-absorbable antibiotic treated group at the end of treatment (32 % versus 77 %), but the effect was lost 7 days post-treatment [134]. A negative result may therefore reflect suppression of the MDRO below the detection limit or temporary suppression rather than decolonisation. An ESCMID guidance document could not find sufficient evidence for a successful therapeutic decolonisation therapy, not with orally non-absorbable antibiotics or any other therapeutic approach [136]. The current knowledge on this topic provided by randomized and observational studies suffers of much heterogeneity between tested populations, used decolonisation therapy, inconsistency in defining and reporting end points and small sample sizes [136], and both large, well-designed RCT as innovative strategies are desperately needed.

Colonisation resistance against (multi drug resistant) Enterobacterales

Modifying the (failing) indigenous intestinal microbiota to prevent or treat gut colonisation with MDRO is an interesting therapeutic intervention, although specific targets are unknown. A healthy indigenous microbiota does not contain an abundance of Enterobacterales. This family of facultative anaerobic Gram-negative bacteria harbours many species capable of MDR carriership. Colonisation resistance against MDRO or Enterobacterales in general, is accomplished by a complex interplay between different species and functions of the host's microbiota. In our recently submitted study on microbiota-associated risk factor for asymptomatic MDRO colonisation study in nursing home residents, several taxa belonging to *Dorea, Atopobiaceae* and *Lachnospiraceae* of the ND3007 group were consistently more

abundant in faeces samples of nursing home residents who were never colonised with an MDRO during a six month time period (submitted, Genome Medicine, Ducarmon et al). At a functional level, many species of the Firmicutes and Bacteroidetes, are capable of metabolizing food fibres to Short Chain Fatty Acids (SCFA's) [137]. Of these SCFAs, butyrate is essential in maintaining host health by providing energy for colonocytes, contributing to the acidification of the lumen, modulating the immune system (maturation and expansion of colonic regulatory T-cells) and affecting diverse metabolic routes in the body (e.g. in liver and brain) [114,116,138-140]. During homeostasis, butyrate-producing bacteria limit the availability of oxygen and nitrate in the colonic lumen through the intracellular butyrate sensor peroxisome proliferator-activated receptor (PPAR-γ) [141]. PPAR-γ represses the gene encoding inducible nitric oxide synthase (*Nos2*), resulting in a lowered nitrate production. Consequently, the bioavailability of electron acceptors is limited, which is normally used for anaerobic respiration and drives an expansion of facultative anaerobes [141-143]. Microbiota induced PPAR-γ signalling also directs the colonocytes towards oxidative phosphorylation and β-oxidation of short and long chain fatty acids, resulting in high epithelial oxygen consumption [141,143]. The consequent epithelial hypoxia helps to maintain a microbial community dominated by obligate anaerobic bacteria [143] or eukaryotes [144]. A depletion of butyrate-producing Clostridia was shown to drive an aerobic luminal expansion of *Salmonella* species [145]. Furthermore, SCFAs at an acidic pH were able to inhibit the replication of *E. coli* and *Salmonella* sp. [146-149], and provide subsequent resistance against colonisation and infection of *Salmonella* following streptomycin treatment [150]. The gut microbiota of nursing home residents carrying an ESBL producing Enterobacterales was indeed characterised by a lower abundance of SCFA producing bacteria [151]. In addition to the above described mechanisms of the healthy microbiota in combat against a perturbed expansion of Enterobacterales, the healthy indigenous microbiota is also capable to inhibit acquisition of antibiotic resistance and horizontal gene transfer [152]. In an *in vitro* model containing a human gut microcosm, the microbiota not only suppressed growth and colonisation of a newly introduced *E. coli* strain, but also prevented it from evolving antibiotic resistant upon exposure to ampicillin. The invading *E. coli* only acquired resistance in the absence of the resident microbial community, even though highly effective β-lactam resistance plasmids were present in the resident microbial communities [152]. In addition, inflammatory responses in the gut can generate transient blooms of Entero-

bacterales in which conjugative transfer occurs at unprecedented rates, as shown by the high rate of conjugative horizontal gene transfer of a resistance plasmid of *Salmonella enterica serovar Typhimurium* to *E. coli* in a mouse colitis model [153].

Innovative prevention of multidrug resistant gut colonisation, and decolonisation strategies

Prevention

An important key to reduce spread of antimicrobial resistance is the prevention of MDRO colonisation in the gut. Restricting the use of (broad spectrum) antibiotics reduces the selection, colonisation and outgrowth of MDRO [154]. This mechanism is well recognized and better known as antibiotic stewardship. Antibiotic stewardship programs are designed to restrict antibiotic overuse and misuse by educating physicians on antibiotic selection, dosage, route of administration and duration of therapy. These programmes have proven their effectiveness and significantly reduce the incidence of colonisation and infections with MDROs and *C. difficile* [155-157]. Accordingly, the improved rational use of antibiotics also reduces sepsis [158] and the overall mortality rates [159]. It seems plausible that at least some of these effects are mediated by the preservation of a diverse and healthy microbiota. Antibiotic stewardship programmes should therefore incorporate consideration for the impact of antimicrobial therapy onto the commensal microbiota [160]. Disruption of the healthy microbiota is in line with use of broad-spectrum antibiotics, and CDI can be considered as surrogate indicator for a disturbed microbiota. However, other infectious complications or diseases associated with a perturbed microbiota may also arise on the short or long term. For example, the use of metronidazole was correlated with intestinal enterococcal domination and subsequent bacteraemia in hematopoietic stem cell transplant patients [161]. In addition, early administration of broad-spectrum antibiotics in allogenic stem cell transplantation patients resulted not only in a decreased abundance of Clostridiales (especially cluster XIVa), but also in a significant higher transplant related mortality [162]. A lower diversity of the intestinal microbiota at the time of neutrophil engraftment was associated with a higher mortality [163]. The early administration of antibiotic therapy active against commensal organisms warrants the use of commensal sparing antibiotics and rapid restoration of the microbiota after cessation of antibiotic therapy. In patients with

rCDI, it was shown that FMT as microbiota restoring therapy (and not anti-CDI antibiotics) lowered the chance on developing a blood stream infection, and subsequent mortality [164].

Therapy

Once the indigenous microbiota failed to provide colonisation resistance against an MDRO and the gut has become colonised, targeting the microbiota could support decolonisation. This decolonisation strategy demonstrated its potential when it was observed that patients receiving an FMT for multiple recurrent CDI, had a significant reduction in the number and diversity of antimicrobial resistance genes after FMT [165-167]. Moreover, not only resistance genes in the microbiota as determined with metagenomics, but also resistance of clinically relevant MDRO Gram negatives decreased after FMT. Data from the NDFB indicate that 50 % of pre-FMT MDRO colonised rCDI patients (ESBL producing or fluoroquinolone and aminoglycoside resistant Enterobacterales), lost the MDRO within three weeks after FMT (preliminary results NDFB and LU-CID, K.E Vendrik and E.M Terveer). This observation adds to various case-reports of patients colonized with ESBL or carbapenemase producing Enterobacterales (CRE) treated and often successfully decolonized by FMT [168-182]. **Chapter 8** describes such a case-report. We experienced that infusion of a healthy donor microbiota into the gut of a patient with normal microbiota diversity did not result in eradication of the ESBL-producing *E. coli*. Possibly specific microbial strains are required, or an improved donor engraftment by antibiotic pre-treatment (further described in paragraph "Optimal donor selection for FMT"). Seven larger case series display mixed results, but varied in study design, patient characteristics and outcome measurement. Of the patients colonised with ESBL, 20 % was decolonised one month after a single FMT, 40 % after two FMTs (n = 15) [171]. Haematological patients (n=25) colonised with either ESBL or carbapenemase producing Enterobacterales (CPE) were decolonised in 60 % of cases one month after FMT [168]. Of note, patients that received antibiotics within seven days after FMT achieved significantly less decolonisation (36 % versus 93 %). Of patients colonised with CRE, decolonisation rates varied from 33 %, 50 % to 80 %, two weeks to four months after FMT [169, 170, 180]. A recent retrospective analysis of CRE and/or VRE colonised patients (n=35) treated with FMT showed that 69 % was decolonised after one year. In addition, microbiota analysis prior FMT could be used to predict the patients response on FMT (or spontaneous

decolonisation), as a higher initial level of Verrucomicrobia and Proteobacteria and lower species richness was observed in the non-responders [183]. These case series should be interpreted with caution as they suffer from publication bias, and also have different study designs with varying pre-treatments, number and application routes of FMT and different follow-up periods. Besides, definitions of gut MDRO colonisation differed and various microbiological tests to detect MDRO in faeces were used. Only one RCT was performed in which 39 adults colonized with ESBL- or carbapenemase-producing Enterobacterales were randomized to either no intervention or a 5-day course of non-absorbable antibiotics followed by FMT. Unfortunately, no statistically significant advantage of FMT was found, though the trial suffered from inclusion of insufficient number of patients [184,185]. Similarly, only small differences in the microbiota composition were observed in the patients after treatment with FMT. Relative to baseline, post-FMT microbiota was significantly enriched in *Bifidobacterium* species and *Collinsella aerofaciens* [186].

The eradication potential of various microbiota modifying agents can be studied via transplantation of a complete ecosystem of a healthy donor. However, in the long run, application of this 'black box' therapy is undesired, and several treatment components merit further research. These are for example "live biotherapeutic products (LBPs)", bacteriocins or other microbial metabolites or bacteriolytic phages. Bacteriophages are highly specific for one bacterial (sub) species, providing a desirable asset in the refine modification of a host microbiota. A number of animal studies showed that bacteriophages can be used as treatment for infections caused by MDRO [187], and also demonstrated potential as eradication strategy for colonisation of MDR Gram negatives (MDR Pseudomonas and MDR uropathogens) of the gut in nematodes and mice respectively [188,189]. The stability of bacteriophages during intestinal passage, their impact on the non-targeted human microbiota, potential side effects and the achievable effect size and duration merit further research [185,187]. An interesting treatment approach is adding bacteriophages to sub-lethal dosages of a non-absorbable antibiotic leading to synergy [190]. The 'phage-antibiotic synergy' is considered an enhanced phage production and accelerated lysis of infected cells, in response to the filamentation of bacterial cells upon exposure to the antibiotic [190]. Other interesting components of FMT acting against MDRO are bacteriocins or (in combination with) live biotherapeutic products. A lantibiotic-producing commensal of the gastro-

intestinal tract, *Blautia producta*, reduced and prevented VRE colonization in man and mice [191]. Another interesting candidate demanding further research is *Lactobacillus*. *Lactobacillus* species in the faecal microbiota of hospitalised patients were associated with resistance to MDRO acquisition during admission [192]. Additionally, mice treated with a combination of 10^{10} CFU *L. plantarum* and *L. acidophilus* were able to eradicate MDR enteroaggregative *E. coli* from the gut [193]. However, up till now probiotics, synthetic bacterial communities or live biotherapeutic products have failed to eradicate intestinal carriage of Gram negative MDROs in human randomized controlled trials. Amongst them, an attempt to eradicate MDROs in ICU patients with *Lactobacillus rhamnosus* [194], a trial to eradicate MDR *E. coli* in long-term care facility residents using *E. coli* Nissle 1917 as probiotic [195], and a combination of *L. bulgaricus*-*L. rhamnosus*-fructo-oligosaccharides failed to eradicate MDR Gram-negative bacilli in hospitalized patients [196]. A live biotherapeutic product of eight living bacterial strains could not eradicate ESBL Enterobacterales in outpatients [197]. The concept of super donors have recently drawn attention in the development of live biotherapeutic products from these donors [198]. The super donors have specific microbial traits and are thereby significantly more capable in treating a specific disease than other donors. Studying these super donors might be crucial in understanding MDRO colonisation resistance and subsequent cure. The concept of super donors for MDRO eradication is currently investigated in a clinical trial by us and others (Davido of the Hôpital Universitaire Raymond-Poincaré in Paris in collaboration with NDFB and Vedanta). For these trials, an FMT donor is selected based on the capability of MDRO clearance in 80 % of the FMT-treated and MDRO colonised mice [199]. Lastly, immunological approaches can be explored as option for MDRO eradication. Vaccination of pregnant cows with the inactivated cells of the globally disseminated and MDR *E. coli* sequence type 131 resulted in highly specific anti *E. coli* 'hyperimmune bovine colostrum'. This colostrum was able to disrupt the intestinal colonization of the ST131 *E. coli* in mice [200].

In conclusion, many therapeutic options are currently explored, but demonstrate high heterogeneity in set-up as well as outcome, and need harmonization. Furthermore, it appears that the concept of FMT for treatment of rCDI is not applicable for decolonization of MDROs. Metagenomic studies could provide answers on the effect of the decolonizing agents on the microbiota composition and dynamics, and should guide the design of future research [136, 186]. Robust and well-designed multicentred

trials to assess the above described innovative therapeutic approaches, with assessment of the optimal pre-treatment, for a larger panel of clinically relevant MDRO's are needed in the future. These studies should also include sufficient long-term follow-up on microbiological and clinical outcomes which assess both adverse events as well as clinical relevance of the decolonization (e.g. reduced MDRO infections and subsequent readmission, fewer long-term complications). Additionally, more fundamental research should be performed using in vitro gut microbiota models to study specific donors, specific microbiota species and networks essential for colonisation resistance for several clinically relevant MDRO's.

Optimal donor selection for FMT

Donors and super donors

A perturbed microbiota has been observed in a large variety of disorders, and FMT as microbiota modulating therapy, is increasingly used in trials not only for intestinal but also for extra-intestinal diseases. The presumed mechanism of action of FMT for other diseases is however likely different than for rCDI and could vary per treatment indication. Though it is generally considered that FMT restores the functionality of a perturbed gut microbiota by engraftment of donor strains, the precise mechanism is probably more complex than a simple replacement of bacterial species. Bacterial networks, metabolites, archaea, viruses, fungi and other eukaryotic microorganisms also influence the composition and function of the microbiota. Corresponding with the variation in gut microbiota composition between healthy individuals (e.g. donors), variability exists in the faecal suspensions used for FMT treatment. Donors of faecal suspensions with a significantly higher success-rate are referred to as super donors. Super donors for rCDI treatment do not appear to exist as no donor related factors attributing to the FMT success could be identified by us (**Chapter 6**) and others [198, 201, 202]. Patients with multiple, recurrent CDI have a perturbed and diminished microbiota diversity [203]. Replenishing this severely reduced diversity with any healthy donor microbiota results in prompt resolution. For other diseases, such as ulcerative colitis super donors do seem to exist [204], but the evidence is sparse. Studying these super donors might be crucial in understanding complex disease pathology and subsequent cure [198]. The question is how to find these super donors?

With respect to donor selection, it is very unlikely that one super donor can cure all microbiota related illnesses [205]. Like our society, a healthy microbiota is diverse. However, this encompasses not only a diverse community within the host, but also between the microbiota's of different hosts. In diseases with a perturbed microbiota, the specific failing network should be restored, and optimal donors could vary per microbiota related disease. For instance a donor for FMT to boost the immune response in patients that show progressive cancer while on checkpoint inhibitors [206, 207] could be very different than the donor needed to abolish the overactive immune system in patients that suffer of grade III/IV toxicity during checkpoint inhibitors [208, 209].

Replenish the beneficial bacteria

Different strategies exist for rational donor selection, depending on the specific disease intended to cure with FMT. A patient can have a decreased load of beneficial bacteria which can be replenished by healthy donor strains *(Figure 3)*. Replenishment is based on supplementation of unique taxonomic or functional deficiencies present in the diseased microbiota [210]. A very successful open-label trial among patients with cirrhosis with recurrent hepatic encephalopathy randomized to receive either standard of care or FMT (with antibiotic pre-treatment), performed rational donor selection. Using microbiome data of hepatic encephalopathy patients and healthy controls a machine learning technique was performed to identify a single donor with the highest relative abundance of *Lachospiraceae* and *Ruminococceae*. FMTs derived from the faeces of this donor significantly reduced hospitalizations, improved the cognition and perturbed microbiota over more than 12 months [211, 212]. If this effect was indeed due to the selected 'super-donor' is questioned [213], since the relative abundance of *Lachospiraceae* and *Ruminococceae* was not significantly different before and after FMT in the FMT-treated patients [213]. A metaproteomic and metabolomic analysis added to the metagenomic data should provide more insights in the functional changes of the group of *Lachospiraceae* and *Ruminococceae*.

An example for which taxonomic selection would be rational is Ulcerative Colitis (UC). The short chain fatty acid; butyrate is important in alleviating inflammatory bowel diseases (IBD) such as UC [214]. A meta-analysis showed a consistent lack of butyrate producing Clostridiales in patients with IBD [215]. A rational super donor would

be a donor with an overabundance of these gut bacteria. This super donor effect was indeed observed in an RCT with FMT for treatment of UC, using five faecal donors. The majority (78 %) of patients who achieved remission received faecal suspensions prepared from one single donor. The other donors were not more efficacious than placebo **[204]**. The super donor contained the highest load of butyrate producing bacteria. Though, in a study combining microbiota data of three RCT's for UC, an abundance of butyrate producing bacteria of the donor was not associated with patient response **[210]**. In addition, host factors are also important in the response to FMT treatment for UC. Younger age, moderate disease severity and endoscopic mayo scores predicted achievement of clinical remission of FMT in patients with active UC **[216]**. This reflects the multifactorial aetiology and treatment of this disease and the challenges of donor selection in the real world. A critical note is that abundance or shortness of a certain group of bacteria in correlation with a specific disease may be an oversimplification. The mucosa associated microbiome and host immune factors may play a more prominent role.

Replacement of the undesired bacteria

A disease could also be mediated by the presence or overabundance of one or more harmful bacteria for which competitor donor strains can be selected *(Figure 3)*. The most straightforward competitors are bacteria that directly inhibit the undesired strain (direct competition), for instance bacteria that produce bacteriocins. An example is the lantibiotic-producing commensal, *Blautia producta*, which demonstrated a reduction and prevention of VRE colonization in man and mice **[191]**. Competitors of undesired bacteria can be identified by another mechanism of colonisation resistance; competitive exclusion. Bacteria occupying the same nutritional or environmental niche can be selected from literature. For instance, *Bacteroides thetaiotaomicron* is a direct competitor of food (carbohydrates) for *Citrobacter rodentium*, a gastro-enteritis pathogen in mice **[217]**. Selection of a donor-mouse containing (high rates of) *B. thetaiotaomicron* to treat a *C. rodentium infection* would therefore make sense. A second illustrative example involves a subgroup of non-alcoholic fatty liver disease (NAFLD) patients, who suffer of auto brewery or gut fermentation syndrome. After a carbohydrate-rich meal, the microbiota of these patients is capable of ethanol production, resulting in an impaired mitochondrial function and subsequent liver injury **[218-220].** Though the pathogenesis of this disease is still unknown, several members of the

microbiota like *Candida* species, *Saccharomyces*, *Enterococcus faecium* or *Klebsiella pneumoniae* have been identified as potential ethanol hyperproducers [221]. Yuan and colleagues show that a high-alcohol-producing *K. pneumoniae* (HiAlc-Kpn-strain) was present in 60 % of individuals with NAFLD in a Chinese cohort, only 6 % of the healthy controls was colonised with this *Klebsiella* [218]. Transfer of the intestinal microbiota from a NASH patient containing HiAlc-Kpn-strain, as well as the HiAlc-Kpn-strain alone into mice, introduced detectable blood alcohol and steatohepatitis. Selective removal of the HiAlc-Kpn-strain (using a bacteriophage) before FMT prevented NAFLD in the recipient mice [218]. Removing this pathology-causing bacterium could thus lead to clinical improvement [218]. Unfortunately, our laboratory could not reproduce these findings of a hyper ethanol producing *K. pneumoniae* in faeces samples of a suspected patient with an auto-brewery syndrome and the Chinese researchers did not provide their strains for further analysis. In patients with auto-brewery syndrome, not only replenishment with healthy microbes but also replacement of the detrimental bacterium is needed. In patients with metabolic syndrome it was already shown this is

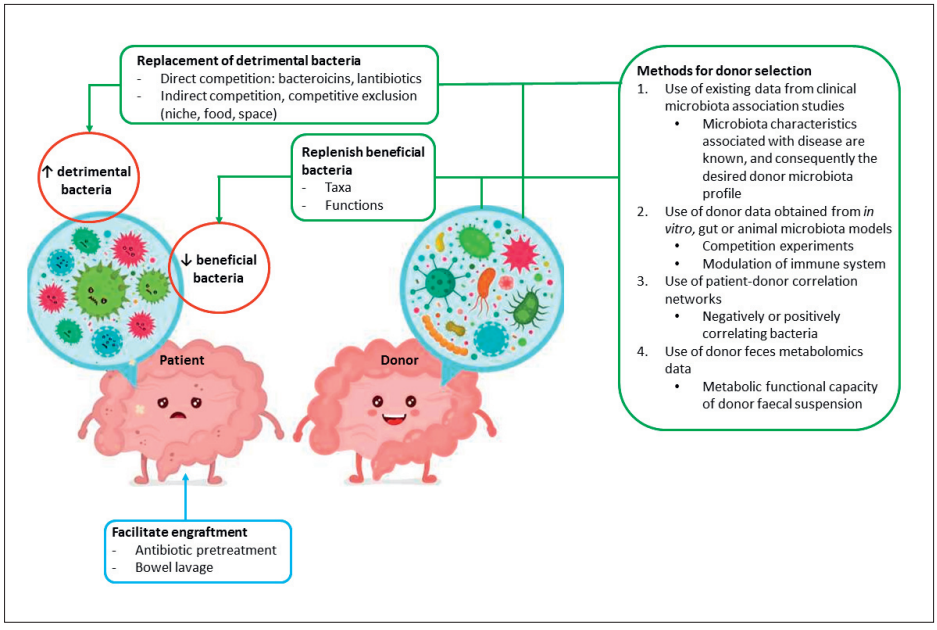

Figure 3. Strategies and methods for optimal donor selection and patient pre-treatment

not always straightforward, as donor strains rather co-colonize than replace similar patient strains [222]. Faecal donors should be screened and excluded when containing (an overabundance of) the undesired bacteria, in addition to positive selection for bacteria known to out-compete the harmful taxa [210].

Proposed methods for optimal donor selection

Option 1: Use of existing data from clinical microbiota association studies

Several approaches can be employed to select optimal donors *(Figure 3)*. De first and most simple technique is selection of donors with a known desired microbiota composition. There are two requirements; First, the microbiota of the donors must be profiled. Metagenomic analysis is preferred, as this provides insight on functional and strain-level associations [223]. Second, the microbiota characteristics associated with disease must be known from epidemiological studies or/and animal experiments.

The NDFB is currently designing a granted FMT pilot study for Parkinson's disease and considers rational donor selection. In this trial the safety and feasibility of FMT in Parkinson's disease patients is assessed. Parkinson's disease is a neurodegenerative disease characterized by neuron degeneration in the central, enteric and peripheral autonomous nervous system. Several mechanisms by which FMT could modulate Parkinson's disease exist. An important factor in the aetiology of Parkinson's is the aggregation of the protein alpha-synuclein [224]. The hypothesis is that under influence of the microbiota, a neurotropic substance, possibly alpha-synuclein, is formed in the gut and transported to the enteric nervous system and brain, via the vagus nerve [225, 226]. Key microorganisms or functions are not yet defined, although the microbiota of Parkinson's patients is in general more pro-inflammatory oriented, with LPS-producing Proteobacteria, and contains less anti-inflammatory butyrate-producing bacteria [227, 228]. The frequently observed obstipation in these patients could however bias the microbiota interpretation, and one could question whether rational donor selection to alter the natural course of disease is appropriate at the moment. Alternatively, the microbiota also seems to play a role in the bioavailability of the primary therapy of Parkinson's disease; levodopa [229, 230]. Bacterial decarboxylases (*tdc* gene) are identified that restrict local (intestinal) and blood levels of levodopa by

conversion to dopamine, which cannot pass the blood-brain barrier [230]. Rational donor selection could therefore be performed with the hypothesis; "FMT from a donor with low amounts of *tdc* genes present in the microbiota will reverse the levodopa resistance by replacement and/or out-competition of *tdc* containing patient strains." The patient will then again respond to levodopa therapy and patients will experience less side-effects (e.g. dyskinesia) due to the stabilised levodopa bioavailability and drug dosing [229]. If this hypothesis holds, the replacement of bacteria carrying *tdc* genes will be most likely based on similar bacteria without the *tdc* gene. If such an effect is found, the next question is whether this replacement will be permanent under continuous exposure to levodopa, or if the patient needs maintenance or sequential FMTs.

Option 2: Use of donor data obtained from in vitro, gut or animal microbiota models

A second strategy involves data obtained from *in vitro* or *in vivo* microbiota models. In close collaboration with Vedanta Biosciences, *in vitro* experiments in mice were performed with faeces of NDFB donors. Antibiotic-pre-treated mice were densely colonized with either a carbapenemase-producing *Klebsiella pneumoniae* or vancomycin resistant *Enterococcus faecium*. The mice were subsequently treated with faecal microbiota from various NDFB donors. Consistent with clinical findings, variability in FMT-mediated decolonization of resistant bacteria was observed. An FMT donor enriched in the microbiota capable to eradicate both MDRO's best, as determined by complete MDRO clearance in 80 % of treated animals [199], was selected for a clinical MDRO eradication trial in kidney transplant patients (trial currently performed, in collaboration with NDFB). Modulation of the immune response by FMT can also be tested in a mouse model. The microbiota plays an important role in the development, training and maintaining of the immune system [231], and the microbiota seems involved in many diseases with an imbalance of pro- and anti-inflammatory responses [232]. Regulatory T-cells are important for the maintenance of intestinal self-tolerance and will likely be important for therapeutically manipulation of IBD [233]. Again, in close collaboration with Vedanta Biosciences, mice sensitive for IBD were colonized with faeces of NDFB donors. The donors which could enhance the regulatory T-cells in the IBD mice the most were selected for an RCT with FMT for UC patients (trial currently ongoing).

Option 3: Use of patient-donor microbiota correlation networks

Another donor selection strategy can be deployed if the microbiota characteristics for a particular disease are not available from human epidemiological studies or animal experiments, but microbiome data of an individual patient is available. The existing microbiome data can be mined to find bacteria that consistently show a negative association with a pathogen or other undesired bacteria. An important assumption with donor selection based on these bacterial networks is that the bacteria that negatively correlate are competitors of the undesired bacterium, rather than that they are both consequence of the underlying disease. The preferred donor should have high abundances of these putative competitors [234]. We studied the microbiota composition of patients with *C. difficile* infection and compared the data with asymptomatically *C. difficile* carriers and healthy controls. The aim was to find special groups of bacteria responsible for progression of a carrier to a diseased state. It was found that the presence of *Eubacterium hallii* and *Fusicatenibacter* may indicate resistance against *C. difficile* colonization and infection, while *Veillonella* may indicate susceptibility [235]. A second example is the role of the microbiota in atopic disease. By mining microbiome data of healthy three months old babies staying either healthy of becoming atopic at the age of 1 year (e.g. asthma, atopic dermatitis, food allergy) Boutin and colleagues showed that this approach can also lead to a potential drug [236]. A machine learning approach revealed a consortium of commensals of the infant gut as candidates for a live biotherapeutic product that could be tested in the future for its potential to prevent the onset or progression of a variety of atopic diseases [236]. A nine-component bacterial community consisting of the following genera was proposed; *Blautia, Coprococcus* (Anaerostipes/Eubacterium_E), *Dorea* (Tyzzerella), *Faecalibacterium*, *Lachnospira*, *Oscillospira* (*Intestinimonas/Flavinifractor*), *Parabacteroides*, *Roseburia* and *Ruminococcus*, and follow-up studies are planned. Although promising, one has to realise with these microbiota association studies that the functional capacity of bacterial genera, species and even strains can be vastly disparate. The cultivation and functional testing *in vitro* and *in vivo* (mouse and human) will be critical for the actual development of a proposed biotherapeutic product

Option 4: Use of donor faeces metabolomics data

A fourth strategy involves measuring metabolomics. One could rationally select a donor based on molecules (butyrate (SCFA) or secondary bile acids) present in

donor faeces that serve as proxy for a 'healthy' metabolic output of the donor microbiota as ecosystem. In patients suffering of multiple recurrent *C. difficile* infections, the metabolomics of bile acids in the faeces clearly differs from healthy donors. In the faeces of rCDI patients, secondary bile acids were absent, whereas primary acids were abundant [237]. FMT promptly normalized the faecal bile acid composition to the healthy donor situation (low primary, high secondary bile acids) [95, 237]. Restoring a disturbance of the capacity of 7α-dehydroxylation of bile acids of part of the microbiota, is not only important in the course of (r)CDI. Many other diseases are influenced by a disturbed bile acid metabolism like liver diseases including cirrhosis, and could therefore be subject of targeted therapy with FMT of a selected optimal donor [211, 238]. A complicating factor is the difficulty of the read-out of the metabolic activity, which is complicated by several individual and environmental factors that influence the absorbance or conversion of the metabolite of interest. For instance the level of bile acids rises after meal [239]. To overcome this bias, the capacity of bile acid conversion of the faecal slurry can alternatively be measured. Ideally the mechanisms of action of the metabolites within complex ecosystems, like the human gut microbiota, must be further explored with a multi-omics approach. The reported integrated use of compositional (metagenomics) and functional (metabolomics and metaproteomics) approaches should preferably be validated with an *in vitro* model to assess the effects of human donor faecal microbiota transplantation to the bile acid pathway. This enables a greater understanding of how variation in the gut microbiota influences host bile acid signatures, their associated functions and their implications for health [240].

Optimizing the patient for FMT to facilitate engraftment

An important step in FMT is to optimize the patient's microbiota to facilitate engraftment of donor strains *(Figure 3)*. This can be performed with bowel lavage to reduce the patient's bacterial load, although its effect has never been compared to placebo [241]. In addition, the undesired strains could be diminished by a (semi-) targeted antibiotic pre-treatment, for example with polymyxin/neomycin for eradication of Gram negative MRDO's as described in **Chapter 8**. Although both in our case-report as well as in a RCT, this combination was unable to eradicate MDROs significantly [173, 184]. Intriguingly, in patients with rCDI, FMT together with vanco-

mycin pre-treatment results in a significant engraftment of donor strains as well as a decline in the number and diversity of antibiotic resistance genes [165, 242]. This decline does not necessarily mean eradication of MDRO's, and the reduction could reflect solely the normalisation of the overabundance of Gammaproteobacteria after FMT in rCDI patients irrespective of vancomycin. On the other hand, the decline in antibiotic resistance genes could also be the result of enhanced engraftment of donor strains capable to compete with the patients' MDRO. Therefore, in contrast to targeting the pathogen with pre-treatment, a more revolutionary idea is to target the indigenous microbiota and create a niche for the donor strains to colonize and compete. Vancomycin is a broad spectrum antibiotic and oral administration results in non-absorbable high intestinal concentrations causing a dramatic decrease of Firmicutes and to a lesser extent Bacteroidetes, the two most important phyla of the indigenous microbiota [73]. Preliminary data on engraftment of live biotherapeutic products in healthy volunteers show that prolonged engraftment is only successful when volunteers are pre-treated with vancomycin (preliminary data of VE303, Vedanta). Additionally, the spore-based microbiome therapeutic SER-287 reported that pre-treatment with vancomycin resulted in a significantly higher engraftment and clinical response in patients with mild-to-moderate ulcerative colitis [243]. Clinical remission was achieved at 8 weeks in 0 %, 13.3 %, 17.7 % and 40 % of patients receiving placebo/placebo, placebo/SER-287 weekly, vancomycin/SER-287 weekly and vancomycin/SER-287 daily respectively [243]. The superiority of vancomycin as pre-treatment enhancing engraftment could however not be confirmed in mice that received donor mice faeces through oral gavage [244]. Surprisingly pre-treatment with polymyxin B resulted in the highest rate of viable donor bacteria in the recipient mice [244]. Of all tested antibiotics, vancomycin, metronidazole and cefotaxime resulted in impaired engraftment efficiency [244]. Lastly, in a proof of principle study on the concept of antibiotic pre-treatment targeting the recipient microbiota, amoxicillin-metronidazole-fosfomycin in combination with FMT (n=27) alleviated the intestinal perturbed microbiota caused by a loss of Bacteroidetes in UC patients better than FMT alone (n=4) [245]. The optimal pre-treatment for bacterial engraftment is currently unknown, and likely varies for the underlying disease and possibly even differs between the specific microbiota modulating therapies (e.g. FMT or LBP).

The relation between *Blastocystis* species and a healthy microbiota

Blastocystis is a genus of a common unicellular obligate anaerobic intestinal parasite in humans and animals that belongs to the stramenopiles, 1 of the 8 major phylogenetic groups of eukaryotes. It is a diverse genus comprising 17 characterized lineages: the so-called subtypes (ST1 – ST17), of which 9 have been reported to occur in the human gastrointestinal tract [246, 247]. *Blastocystis* sp. carriage is very common but varies globally, from 0.5% in Japan to 100% in Senegal and 30–50% in Europe [18, 248-250]. An interesting finding in our NDFB patient cohort (**Chapter 7**) was that none of the rCDI patients carried *Blastocystis* species [251]. Low *Blastocystis* sp. colonization rates in diseased patients were previously also reported in IBD patients [18-20]. IBD and rCDI patients have a very disturbed microbiota in common. It is unknown if the association between a disturbed microbiota and low *Blastocystis* sp. colonization results from the inability of *Blastocystis* to survive in a disturbed environment. Homeostasis of the microbiota is associated with butyrate-producing bacteria, resulting in oxygen consumption by the colonocytes (for more information, see section "Colonisation resistance against (multidrug resistant) Enterobacterales", this thesis) [141, 143]. The subsequent epithelial hypoxia helps to maintain a microbial community dominated by obligate anaerobic bacteria [143], or oxygen sensitive eukaryotes like *Blastocystis* species [144]. The result of antibiotic related depletion of butyrate-producing bacteria can be observed in some perturbed microbiotas, such as rCDI and IBD, where a shift occurs from obligate anaerobic bacteria belonging to the phyla Firmicutes and Bacteriodetes to the facultative (an)aerobes of the phylum Proteobacteria [242, 252]. The presence of *Blastocystis* in half of the patients after transfer of healthy donor faeces (FMT), could reflect reestablishment of a healthy microbiota after FMT. The second theory encompasses a top-down control of the microbiota by *Blastocystis*, the parasite itself influences the composition of the microbiota by predation or ecosystem management and thereby creates a more diverse microbiota [27]. Evidence of the predatory capacity of *Blastocystis* on bacteria is shown by the capability of bacterial engulfment [253] and the low frequency of the ameboid form in axenic cultures. In this case, the transfer of *Blastocystis* sp. by FMT could enhance the microbial diversity of the patient more than non-*Blastocystis* containing faecal suspensions would.

In general, the concept is increasing that *Blastocystis* is a marker for a healthy microbiota [18, 22-27]. We showed that FMT containing *Blastocystis* ST1 or ST3 did not result in an altered treatment efficiency or gastrointestinal symptomatology (**Chapter 7**). Therefore, *Blastocystis* ST1 and ST3 should be deleted as donor exclusion criterion, although screening and long-term follow-up of the patients is preferred. Additionally, FMT trials for rCDI and other indications should allow *Blastocystis* positive donors and test whether this leads to a higher efficiency to cure disease with FMT.

Quality assurance of faecal suspensions

With the emergence of FMT as new treatment approach, stool banks are needed to provide ready-to-use donor faecal suspensions that are produced in a standardized way [254]. A donor faecal suspension is however not a standardized drug that is produced in a factory, but a highly diverse and donor-specific microbiota in its broadest sense, also known as substance of human origin (SoHo; blood, tissues, cells and organs) [255]. This implies that faecal suspensions and subsequently stool banks require (inter)national guidance of quality and safety measures, comparable of other SoHo therapies [255]. Significant advantages of centralized donor screening and production of donor faecal suspensions are the possibilities to provide quality assurance, standardisation of manufacturing and appropriate monitoring of unexpected adverse events. The current FMT product manufacturing protocols are for a large part based on expert opinion [256, 257], and optimized for treatment of *C. difficile*. The FDA recently published "Regulatory considerations for FMT products", in which it is stated that the stability and viability testing should be considered for FMT products used for clinical trials [258]. In Europe, the Guide to the quality and safety of Tissues and Cells for human application (Tissue Guide) of the European Council includes a chapter about FMT, that is currently revised and may serve as a reference for quality assurance of FMT in Europe.

Viability of anaerobic bacteria during processing and manufacturing

Faecal suspensions for FMT are most often produced in ambient air (aerobic preparation). A recent *in vitro* study applied Propidium Mono Azide (PMA) to measure the viability of bacteria after aerobic and anaerobic processing. PMA is a fluorescent dye which selectively enters cells with a compromised cell membrane. Upon exposure

to light, PMA covalently binds to DNA in these cells or naked DNA, thus leaving only viable cells available for PCR amplification [259]. An optimization of this method to apply on stool samples has been developed [260]. The study of Papanicolas and colleagues showed that aerobic processing decimated the yield of delicate obligate anaerobic bacteria like *Faecalibacterium* sp., *Eubacterium rectale*, *E. halli*, *Subdoligranulum* sp., Anaerostipes, *Megamonas*, *Bifidobacterium* and *Roseburia* up to 12-fold [259, 261]. Other taxa were found to be more oxygen resistant such as *Bacteroides*, *Parabacteroides*, *Barnsiellaceae* or *Rikenallaceae* [261]. In the case of rCDI, this aerobic degradation has little or no impact on clinical efficacy as studies using anaerobically produced FMT suspensions do not report a significant increase in rCDI cure rate (cure rate of 80 %) [262-264]. For other indications such as IBD or hepatic encephalopathy, where the therapeutic component is poorly understood, variation of the number of living anaerobic bacteria could theoretically have significant effect on the clinical outcome. In the ulcerative colitis RCT of Moayyedi and colleagues a super donor with high levels of butyrate-producing bacteria was found [204]. These butyrate producing bacteria often belong to the Firmicutes, a phylum disproportionally affected by oxygenic stress [261, 265]. Manufacturing faecal suspensions in ambient air resulted in a more than 2.5-fold reduction in relative abundance of butyrate-producing bacteria. Consequently this impacted the level of the gene encoding a terminal enzyme in the dominant pathway of butyrate biosynthesis (butyryl-CoA:acetate CoA-transferase gene), and subsequent post-fermentation of butyrate levels was reduced with approximately 50 % [259]. In contrast to oxygen exposure, lag time (time between defecation and processing) and freeze-thaw steps didn't seem to alter the living microbiota much, both in absolute amount as well as the composition [259, 261, 266]. Altogether the loss of butyrate-producing, and obligate anaerobes combined with the relative overabundance of oxygen tolerant bacteria could potentially transform a healthy donor microbiota into faecal suspensions containing a microbiota profile more closely resembling those of the patients.

Viability of anaerobic bacteria during storage

Two RCTs and one meta-analysis showed non-inferiority and comparable cure rates for the treatment of rCDI with fresh or frozen faecal suspensions (stored at -80°C for up to 30 days) [267-269]. Use of a frozen faecal suspension allows storage at -80°C for a longer period of time until the donor has been retested prior to actual use of the

donor faecal suspension. This lowers the risk of transferring transmissible diseases by bypassing the window of detection phase of some transmissible infections (e.g. HIV, Hepatitis C). Having well-screened donor faecal suspensions in storage will allow a more rapid and safe transplantation when needed, bypassing the logistical difficulties of preparing a fresh FMT suspension. In addition, it allows extended screening and selection of preferred donors and specific faecal suspensions that are required for FMT for non-CDI indications. To prepare frozen suspensions, a cryoprotectant should be added prior to freezing. In general, the cryoprotectant glycerol is used in a final concentration of 10 to 15 %. Cryopreservation is a process of preservation of the biological and structural functions of tissues or cells when cooling to sub-zero temperatures [270]. Viability of six representative groups of faecal bacteria after six months of storage at -80°C in normal saline with 10 % glycerol did not differ from baseline, whereas viability was reduced in suspensions stored with saline alone. Especially, the aerobes, total coliforms and lactobacilli were significantly reduced by >1 log in the faecal suspension stored without glycerol [263]. In addition, the authors conclude that the protective effect of glycerol outweighs the presumed detrimental osmotic effect of glycerol on living cells. Long-term storage should be at -80°C or lower to prevent sample degradation. High cure rates have been reported with frozen FMT suspension stored up to two years -80°C [16, 262, 263, 266, 271-275]. In fact, both the NDFB and OpenBiome concluded that storage duration did not impact the clinical effectiveness of FMT for rCDI patients (**Chapter 6**, [276]). Whether a shelf-life of two years is also applicable for other diseases remains to be investigated, as *in vitro* studies suggest long-term storage does seem to impact some bacteria more than others. To test to what extent donor microbiota communities are affected by the manufacturing and storage procedures at the NDFB, a culturing pilot study was performed by the NDFB in close collaboration with Vedanta Biosciences. Donor faeces was collected and divided in two aliquots, one placed in an anaerobic chamber, the other processed aerobically and frozen within 30 minutes. Both aliquots were serial diluted and inoculated onto eight different selective and non-selective media. PCR and Sanger sequencing was performed on 1288 picked colonies. A general 10-fold loss in cultivability of anaerobic bacteria was found during processing in combination with storage at -80°C and subsequent thawing. Bacillus species, and *Anaerostipes hadrus* were identified [277]. In a study that subjected fresh and frozen faecal microbiota suspensions to stress conditions that bacteria may undergo after transplantation in the

human gut with FMT, the results showed that the abundance of Bacteroidetes decreased with longer storage times [278], in particular when stored beyond 15 months of storage at -80°C (with glycerol). In contrast, Firmicutes showed good resistance to a harsh DNA extraction protocol, including Proteinase K treatment (solubilizes solid human tissues, disrupts biofilms), DNAse treatment next to a chaotrophic agent (guanidine hydrochloride; disrupts human cells and also has affinity for Gram negative bacteria). More specifically, Operational Taxonomic Units (OTUs) of butyrate producing bacterial species, showed relatively little changes of relative abundance when frozen samples were compared to fresh samples [278]. The question remains if this pre-treatment of the faecal suspensions represents the *in vivo* conditions after transplantation. However, an *in vivo* mice experiment showed similar results. In this study, the viability was assessed using 16S rRNA analysis after PMA pre-treatment in fresh faeces compared to faeces stored at -20°C. The viability of frozen faeces was comparable with fresh faeces [279], but after transplantation in mice, some bacterial taxa were attenuated in enteric colonization ability when stored frozen. Bacteroidetes, next to Actinobacteria and Deferribacteres showed less resilience or colonization ability after freezing at -20°C for more than 1 month [279]. A second mice study used a complementing technique to test the viability of transplanted microbiota by labelling the gut microbiota *in vivo* of donor mice with a fluorescent marker. After FMT of the fluorescent donor microbiota, the recipient mice received a second fluorescent marker with another colour. The viable (metabolic active) portion of the donor microbiota incorporates both markers and can readily be distinguished from dead donor bacteria. 16S rRNA analysis indicated that several bacterial genera were enriched, including Gammaproteobacteria, *Clostridium XlVb* and *Butyricicoccus*. Although FMT in this study is probably less efficient because the donor microbiota was administered by gavage and therefore did not bypass the acid stomach, the viability of donor *Clostridium XlVb* and *Butyricicoccus* strains is encouraging, since these are generally considered to be beneficial to the host [138].

Proposed quality control for viability and stability of faecal suspension microbiota

Some of the studies show a substantial donor variation in the viable component of faecal suspensions affected during manufacturing and storage [138, 259, 278, 279]. This could be explained by individual variation in microbiota composition, resulting in

a different vulnerability of the microbiota to stressors as oxygen exposure or freezing. The composition and function of a healthy individual's microbiota is in general stable, and resilient to most perturbations (low intra-donor variability) **[280]**. However, minor changes in environmental factors such as diet, medicine use, season, travel or household contact can have large effects on the microbiota **[281-283]**. The potential differences in intra- and inter-donor stability and viability during processing indicate the need for viability assays performed as quality control. Promising as relatively quick and less expensive screening tool for viability of the microbiota of a faecal suspension is a combination of staining (for instance a classical Live/Dead stain (based on fluorescein diacetate (FDA) and propidium iodide (PI), which stain viable cells and dead cells, respectively) and flow cytometry. It has the potential of facilitating the analysis of complex ecosystems through visualizing the changes in the dynamics of bacterial communities **[284]**. This can be combined with periodic deeper microbiota assessment with a subset of different methodologies to provide more detailed information. Analysing the microbiota of sequential faecal samples with a combination of culturomics, 16S analysis and flow cytometry showed that the various methods were additive to each other **[205]**. In addition, culturomics showed the relevance of using sequential samples as many bacteria were found irregularly as the faecal microbiota may, to some extent, change daily **[205, 285]**. In the end, investigating the functional microbiota, for instance by means of the pool of genes is the most important, as the functional traits of the microbiota should be maintained, and are not necessarily provided by the same organisms **[286]**. Further research should focus on the best strategy for quality control of faecal suspensions for FMT treatment. Most likely this will involve multiple of the above-mentioned techniques once every 6 months to 1 year, in addition to a more frequent performed basic microbiology viability and stability check which encompasses culturing of several indicator anaerobes. Careful clinical follow-up is the ultimate quality control and should be organised by stool banks to establish the safety of their protocols.

Future of stool banking

Stool banks were initiated to implement safe and cost effective FMT. Gradually, stool banks became expertise centres with experts in the fields of microbiology, gastroenterology, infectious diseases, biobanking, data science, microbiome research

and pharmacy. This may result in multidisciplinary trials addressing the effects of microbiota modulating therapies in a wide range of disorders. Stool banks also enable fundamental research addressing both pathogenesis, functional microbiota networks, and mechanism of action to develop new treatment concepts.

An interesting new application of the experience and expertise of stool banks is the banking of faeces for auto-transplantation. In case of an expected and undesired major change of the gut microbiota such as patients undergoing a stem cell transplantation, a stool bank can facilitate with storage of pre-event faeces. A second interesting application is the banking of faeces of patients that respond well to anti-cancer therapy. The NDFB will participate in an RCT phase Ib trial in metastatic melanoma patients refractory to immune checkpoint inhibitors (ICI) receiving either an FMT of an ICI responder or non-responder patient (prof. J Haanen, Oncology LUMC and AvL). The NDFB will in this case not provide faecal suspensions of healthy donors but collaborates with the knowledge on microbiota modulating therapies (e.g. patient-donor selection and screening, manufacture of faecal suspensions, biobanking an preservation of the microbiota, selection and pre-treatment of patient, FMT treatment itself and follow-up of the patients. The rationale for this trial is that pre-clinical data indicates that the gut microbiota controls the immune response and subsequent response to ICI. The use of antibiotics within the first 3 months prior to initiation of ICI has been demonstrated to negatively influence the treatment response **[206, 287, 288]**. Tumour bearing mice demonstrate that FMT of responder-patients can improve the anti-tumour immune response, and when combined with ICI can improve outcome **[206, 207]**.

Banking faecal microbiota suspensions for FMT is a new research field and is constantly evolving and developing. Already in the beginning, FMT was recognised to have great potential to cure microbiota related diseases. The strength of this treatment is the transplantation of a complete ecosystem. Nevertheless, the weakness of this therapy also lies in transplanting a complete, but uncontrolled, unstandardized and not fully understood ecosystem. An undesired pathogen or disease trait could be co-transplanted. To limit risks, standardisation of working processes of stool banks was established and standard operating procedures were formulated addressing; the recruitment, selection and screening of donors, processing and manufacturing of the

donor faeces and storage and distribution of frozen faecal suspensions, together with selection, treatment and follow-up of patients both on institutional and national as international level (**Chapter 5**) [241, 257]. Recommendations are regularly updated and adapted to new situations, such as the recent new advices to screen donors for the presence of enteropathogenic *E. coli*, MDROs and SARS-CoV-2 [289, 290]. The risk of infectious complications after FMT depends in part on appropriate donor screening. This may even be more important for severely immunocompromised patients, as suggested by the cases where transfer of MDRO by FMT in neutropenic patients resulted in sepsis and death [291]. Following these cases and the subsequent FDA warning, the NDFB evaluated their screening protocol, with periodic screening every three months and targeted rescreening after foreign country visits. Although 25 % of active donors became MDRO positive at some point during their donation activities, the current NDFB screening protocol did not result in approval of MDRO-positive faecal suspensions for FMT treatment (K.E.W. Vendrik et al., Lancet Infectious diseases, in press). However, although the residual risk of transmission of MDROs appears acceptable for most patients, this risk appears not acceptable for severely immunocompromised patients based on the above-mentioned cases. Therefore, the NDFB performs direct screening of suspensions used for immunocompromised patients [292]. These studies are a step towards a more evidence-based way of donor screening, and stool banking. Setting up a national or even international registry both for donor and patient follow-up data would lift FMT as quality-assured treatment strategy to the next level.

Future of microbiota modulating therapies

In recent years FMT has been implemented worldwide as effective rescue therapy for patients with multiple recurrent CDI, with cure rates of approximately 85 % [83, 293-295]. Transplanting faecal microbiota of a healthy donor with the aim to restore a patient's perturbed microbiota appears also promising for several other disorders, such as ulcerative colitis, hepatic encephalopathy and a subset of inflammatory bowel syndrome patients [211, 296-299]. Furthermore, many of the previously discussed indications are interesting and merit further research. It is illustrative that while writing this discussion, more new indications and applications pop-up as potential target for microbiota modulating therapy, such as FMT

for immune modulation of patients suffering of severe COVID-19 [300], or patients with systemic sclerosis [301] and patients with alcohol use disorder [302].

Faecal suspensions for FMT contain a highly diverse microbial ecosystem. Because of the unstandardized nature of this treatment, and potential risk of transfer of unrecognized pathogens or disease traits, a more controlled and standardised treatment is desired in the future. Most newly developed microbiota modulating therapies involve synthetic bacteria or bacterial communities (live biotherapeutic products). In the future well-regulated and characterized live biotherapeutic products are preferred over probiotics which are not regulated and can be sold without quality check as food additive. Most probiotic companies do not characterize the microorganisms or assess the presence of AMR and virulence genes. Recently the scientific community was startled by the finding that the probiotic *E.coli* Nissle 1917 contained colibactin encoded by a pks island [303]. Colibactin is tumorigenic in murine models and more prevalent in patients with colorectal cancer compared to healthy controls [304]. In the past, an unexpected increased mortality caused by the probiotic Ecologic 641 (a mixture of *Lactobacillus*, *Lactococcus* and Bifidobacteriae) was reported in patients with acute pancreatitis participating in the PROPATRIA study [305]. This was explained by the finding that the disrupted intestinal barrier of the patients with concomitant organ failure, in combination with the probiotic strains, resulted in increased bacterial translocation and enterocyte damage, with subsequent mortality in 16 % versus 6 % in the placebo group [306]. This illustrates that selecting an unbalanced mix of several "beneficial" strains, without complete understanding of the function, effects and their interaction with and within the host is not without risks. In a landmark paper on treatment and prevention of antibiotic induced perturbation of the microbiota, it was shown that a commercially available 11-species probiotic markedly delayed indigenous gut mucosal reconstitution after antibiotic exposure [307]. Compared to spontaneous post-antibiotic recovery, the microbiome reconstitution (both in composition as well as in transcriptome) was not only delayed, but also remained incomplete by the end of the intervention period (day 28) or five months after probiotics cessation. An auto-FMT induced rapid and near-complete recovery within eight days [307].

Many host-microbiota interactions pertaining with human health and disease are mediated by metabolites. These metabolites can be secreted, degraded or modified

by the gut microbiota or the host, or given as encapsulated therapy. By bypassing the transfer of live bacteria (e.g. in a bacterial mix or FMT), but instead provide metabolites, some of the caveats of current microbiota modulating therapy can be overcome, such as transfer of opportunistic pathogens or unwanted effects on unrelated conditions, or the individual variation in colonization resistance and engraftment of donor strains **[308]**. Microbiota associated metabolites of interest are short- or long-chain fatty acids, bile acids, vitamins or polysaccharides. This therapy aims at impacting their downstream signalling pathways when relevant to pathogenesis of disease. Microbial molecules of therapeutic potential are not limited to secreted metabolites, but may also include cellular components, such as membrane proteins **[117]** or even sterilised bacteria **[309, 310]**.

During the establishment of the NDFB in 2015, it was believed that other microbiota modulating therapies (for example live biotherapeutic products) would have replaced FMT for rCDI within five to ten years. To date, not a single microbiota drug has shown significant and relevant treatment outcomes for rCDI **[101]**. Rebuilding a well characterised synthetic microbiota community with the capability of resilience to *C. difficile* infection and relapse is much more difficult than previously thought. Once such strategies are of proven benefit in the future, this may result in effective and safe new drugs to cure and prevent rCDI and replace FMT as treatment approach. At present, transplanting a healthy faecal microbiota with the aim to restore a patient's perturbed microbiota remains the standard therapy for patients with multiple recurrent CDI, and is promising and performed in research setting for many other diseases. For development of more sophisticated precision microbiota therapeutics, FMT will pave the way by providing mechanistic insights in the effects of the transplanted microbiota on a specific disease. In the future, preferably an arsenal of several precision microbiota therapeutics would stand to our disposal which should be administered on a tailored basis as a personalised microbiota modification treatment.

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Nederlandse samenvatting

Antibiotica hebben de mensheid een ongekende slagkracht tegen bacteriële infecties geboden. Hierdoor veranderde het beloop van vele infectieziekten, en worden jaarlijks miljoenen levens gered. De opkomst van antibiotica is misschien wel één van de meest belangrijke ontwikkelingen geweest die onze huidige hoogwaardige geneeskunde mogelijk heeft gemaakt. Zonder antibiotica is een open-hart operatie of beenmergtransplantatie bijvoorbeeld een stuk risicovoller, zo niet onmogelijk. De andere kant van de medaille is een verstoring en verarming van de menselijke bacteriële darmflora (darmmicrobiota), en het ontstaan van bacteriën met antimicrobiële resistentie. Een verstoring in de microbiota resulteert in een verhoogde vatbaarheid voor een darminfectie met *Clostridioides difficle* en mogelijk voor diverse Westerse, levensstijlgeassocieerde ziekten zoals suikerziekte, kanker of inflammatoire darmziekten (colitis ulcerosa). De Wereldgezondheidsorganisatie (WHO) verklaarde antimicrobiële resistentie als één van de grootste uitdagingen voor de volksgezondheid van dit tijdperk, omdat het een goede behandeling van veelvoorkomende bacteriële infecties belemmerd. Naast multiresistente *Neisseria gonorrhoea* en *Candida auris* worden multiresistente Gram-negatieve bacteriën (carbapenem resistente Enterobacterales) en *C. difficile* door het Centers for Disease Control and Prevention (CDC) als meest urgente bedreigingen bestempeld.

C. difficile en multiresistente Gram-negatieve bacteriën (BRMO: bijzonder resistente micro-organismen) gedijen goed in een samenleving waar breedspectrum antibiotica frequent gegeven wordt. Voor beide micro-organismen speelt dragerschap in het maag-darmkanaal een belangrijke rol in de ontstaanswijze van infectie, oftewel pathogenese. Een aanzienlijk deel van gezonde mensen (2-10 %) is asymptomatisch drager van *C. difficile* en/of een BRMO, maar bij slechts een beperkt gedeelte leidt dit tot ziekte. Bij gezonde asymptomatische dragers onderdrukt een complex samenspel van de darmmicrobiota, door competitie om voedsel en plaats, de uitgroei van *C. difficile* of BRMO. Dit mechanisme staat bekend als kolonisatie resistentie. Na antibioticagebruik kan de microbiota tijdelijk ernstig verstoord zijn, waardoor de kolonisatie resistentie afneemt en *C. difficile* kan uitgroeien tot grote hoeveelheden en toxines produceren die leiden tot een ontstekingsrespons met diarree en soms ernstige ontsteking van de darm (pseudomembraneuze colitis of zelfs toxisch megacolon).

Het doel van dit proefschrift is beter inzicht te verschaffen in de rol van de microbiota in verdediging tegen infecties met multiresistente Gram-negatieven en *C. difficile*. Het eerste deel beschrijft de epidemiologie (het vóórkomen), en de detectie van *C. difficile* en BRMO dragerschap in verschillende populaties. Het tweede deel richt zich op de behandeling van dragerschap of infectie met deze micro-organismen, middels herstel van de verstoorde darmmicrobiota door transplantatie met gezonde donormicrobiota, oftewel fecale microbiota transplantatie (FMT).

Deel I: Nieuwe inzichten in de epidemiologie van *Clostridioides difficile* en multiresistente Gram-negatieve bacteriën

Lang werd gedacht dat infecties met *C. difficile* verworven werden in het ziekenhuis door (nosocomiale) overdracht via symptomatische patiënten. Recent verschenen er enkele publicaties waarin werd beschreven dat de overdracht van *C. difficile* naar de ziekenhuisomgeving, gezondheidsmedewerkers en andere patiënten ook wordt gefaciliteerd door patiënten die *C. difficile* asymptomatisch met zich mee dragen. Herkenning van deze asymptomatische dragers is daarom essentieel om de omvang van C. difficile verspreiding te bepalen en mogelijk te verminderen. De laboratoriumtechnieken om asymptomatisch gekoloniseerde patiënten te herkennen, zijn onderzocht in **hoofdstuk 2**. In de feces van patiënten zonder klachten van diarree en afgenomen bij opname in één van drie grote ziekenhuizen in Nederland, werden drie verschillende diagnostische *C. difficile* testen vergeleken met de toxigene kweek als gouden standaard. De feces werd getest met een enzym-linked fluorescent assay (ELISA) gericht op glutamaat dehydrogenase (GDH) en twee moleculaire testen gericht op het toxine gen (de commerciële artus PCR gericht op toxine A en B, en een *in-house* PCR enkel gericht op toxine B). Uit deze studie bleek dat 5.1% van de patiënten bij opname in het ziekenhuis een *C. difficile* bij zich droeg; 3.1% was drager van een toxine producerende *C. difficile*. In deze laag endemische setting van *C. difficile* dragerschap kunnen alle drie de testen worden gebruikt als eerste screeningstest, gelet op de hoge negatief voorspellende waarde. Net als bij de diagnose van patiënten met een *C. difficile* infectie (CDI), is de positief voorspellende waarde bij lage prevalentie (weinig vóórkomen) niet optimaal. Een discrepantieanalyse liet zien dat het merendeel van het relatief kleine aantal fout-positieve testen niet kon worden bevestigd na herhaling. Nu we weten dat *C. difficile* dragers goed gedetecteerd kunnen worden, rijst de vraag wat te

doen met een positieve bevinding. Alhoewel dragers een bron voor verspreiding van *C. difficile* kunnen zijn, en in een Canadese studie het isoleren van dragers leidde tot een vermindering van in het ziekenhuis ontstane CDI, werd dit voordeel in een Nederlandse studie (2016-2018) niet bevestigt. In Nederland worden *C. difficile* dragers niet actief opgespoord. Indien dragerschap wordt gevonden in patiënten met diarree door een andere oorzaak dan CDI zal de patiënt echter wel in isolatie worden gelegd.

Ook voor BRMO's speelt nosocomiale transmissie door asymptomatisch dragers een significante rol. Preventie van BRMO-transmissie binnen ziekenhuizen wordt nagestreefd door geselecteerde patiëntgroepen met een hoog risico op dragerschap te screenen en zo nodig te isoleren. Eind 2015 werd in China een nieuw soort colistine resistentie (*mcr-1*) bij Enterobacterales ontdekt in dier en mens. Colistine is een reserve-antibioticum dat ingezet kan worden ter behandeling van BRMO's. Nieuw aan deze vorm van resistentie is dat het plasmide gemedieerd is. Dit betekent dat de overdracht van de resistentie tussen verschillende bacteriën veel gemakkelijker zal gaan. Momenteel zijn tien *mcr*-genen (*mcr-1* tot *mcr-10* ontdekt in verschillende Enterobacterales. Om het risico van introductie van *mcr* door asymptomatisch dragers in ons academisch ziekenhuis in kaart te brengen, werd in **hoofdstuk 3** de prevalentie van dragerschap bepaald van patiënten die opgenomen werden in ons ziekenhuis. Twee van de 576 (0.35%) patiënten werden positief getest op *mcr-1. Mcr-2* werd niet gevonden. Ten tijde van het uitvoeren van deze studie waren epidemiologische gegevens over de prevalentie van dragerschap bij gezonde individuen niet beschikbaar, maar enkele recente onderzoeken van het RIVM bevestigden het lage percentage dragerschap (0.5%) van *mcr-1* positieve bacteriën bij gezonde mensen. De verspreiding van *mcr* vanuit de samenleving naar de ziekenhuisomgeving in Nederland is dus laag. In ons onderzoek werd een fenotypisch colistine gevoelige *mcr-1* bevattende *Escherichia coli* gekweekt. De discrepantie tussen de fenotypische en moleculaire bepaling bleek te berusten op een defect *mcr-1* gen (1329kb transposon in het *mcr-1* gen). Het resistentiegen kwam hierdoor niet tot uiting, waardoor de bacterie gevoelig bleef voor colistine. Dit onderstreept het belang van fenotypische bevestiging na moleculaire screening op een resistentietargetgen.

Verpleeghuisbewoners hebben een aantal risicofactoren die kolonisatie en infectie met *C. difficile* en BRMO bevorderen. Veel bewoners komen veelvuldig in het zieken-

huis of hebben andere zorgcontacten. Daarnaast is er vaak intensief contact tussen de medebewoners en zijn sommige bewoners fecaal incontinent, waardoor verspreiding van BRMO's wordt vergemakkelijkt. In **hoofdstuk 4** wordt beschreven dat risicofactoren voor dragerschap en infectie frequent aanwezig zijn bij Nederlandse en Ierse verpleeghuisbewoners, maar dit leidde niet tot een hoge prevalentie van BRMO's; respectievelijk 9% en 11% van de Nederlandse en Ierse verpleeghuisbewoners was drager van een Extended Spectrum Beta Lactamase (ESBL) producerende *E. coli*. Geen van de patiënten had een carbapenemase producerende Enterobacterales, vancomycine resistente enterokok of *C. difficile* in de ontlasting. Met behulp van de DNA-typeringstechniek 'core-genome multi locus sequence typing' (cgMLST) werd kleinschalige verspreiding van BRMO's tussen verpleeghuisbewoners van eenzelfde afdeling binnen het Nederlandse verpleeghuis, en tussen verschillende afdelingen in het Ierse verpleeghuis aangetoond. Dit verschil in transmissie van BRMO's tussen beide landen zou een weerspiegeling kunnen zijn van verschillen in de werkwijze en de infrastructuur van de onderzochte verpleeghuizen in Ierland en Nederland, zoals bijvoorbeeld meerpersoonskamers en een andere invulling van de activiteiten in gemeenschappelijke ruimtes.

De hoofstukken van deel I van dit proefschrift beschrijven dat asymptomatische kolonisatie van BRMO's en *C. difficile* een reservoir kan vormen voor nosocomiale transmissie. De prevalentie ervan is in Nederland echter (nog) laag, en de huidige praktijk van detectie en preventie in Nederland lijkt dus effectief.

Deel II: De oprichting van een Nederlandse Donor Feces Bank om microbiota transplantatie te faciliteren

Het doel van feces microbiota transplantatie (FMT) als behandeling van terugkerende *Clostridioides difficile* infectie (rCDI) is het verbeteren van de microbiota samenstelling middels het inbrengen van gezonde donormicrobiota, waardoor de kolonisatie resistentie tegen *C. difficile* hersteld wordt. Hierdoor kan *C. difficile* niet opnieuw uitgroeien, toxinen produceren en de symptomen van een infectie veroorzaken. FMT is inmiddels in nationale en internationale richtlijnen als gevestigde behandeling voor mensen met rCDI opgenomen, en wordt ook veelvuldig onderzocht als behandeloptie voor andere ziekten. Fecesbanken die zich kunnen richten op zowel

de standaardisatie, veiligheid, kwaliteitswaarborging, beperking van kosten én doelmatigheid zijn hierdoor in toenemende mate van belang. Fecesbanken leveren kant-en-klare fecessuspensies voor behandeling van rCDI patiënten in het eigen, lokale ziekenhuis. **Hoofdstuk 5**, 'How to run a stool bank' beschrijft de oprichting van de Nederlandse Donor Feces Bank (NDFB). Het geeft inzicht in de zeer strenge screening en selectieprocedure van donoren en de bereiding, opslag en toediening van fecessuspensies. De eerste patiëntengroep die met door NDFB uitgegeven fecessuspensies werd behandeld, is gevolgd om de kwaliteit van onze behandeling te evalueren. In vergelijking met andere fecesbanken of FMT-expert centra heeft de NDFB een relatief hoog rCDI genezingspercentage van bijna 90 % na twee maanden en van meer dan 70 % na een gemiddelde follow-up van 42 weken (**hoofdstuk 6**). Dit hoge succespercentage wordt waarschijnlijk mede verklaard door de inspanningen van ons multidisciplinaire FMT-expertpanel. Dit expertpanel bestaat uit een arts-microbioloog, maag-darm-leverarts en internist gespecialiseerd in infectieziekten, en bespreekt de indicatie van de patiënt aangemeld voor FMT, en adviseert tijdens de behandeling en follow-up van de patiënten. Deze strategie resulteert in een effectief, veilig en doelmatig gebruik van FMT voor de behandeling van rCDI.

Bij donorscreening zijn nog een aantal niet opgeloste microbiologische vraagstukken. Gezonde donoren die bijvoorbeeld drager zijn van de parasiet *Blastocystis* worden uitgesloten van FMT-donorschap. Dit resulteert in exclusie van een aanzienlijk deel van gezonde donoren (30-50 %). Het is de vraag of dit gerechtvaardigd is omdat het ziekmakend vermogen (entero-pathogeniciteit) van *Blastocystis* niet overtuigend aangetoond is. De in het verleden veronderstelde entero-pathogeniciteit is gebaseerd op anekdotische casusbeschrijvingen en retrospectieve studies. Interessant is dat recente literatuur juist een lage prevalentie van intestinaal *Blastocystis* dragerschap laat zien bij diverse aandoeningen geassocieerd met een verminderde diversiteit van de darmmicrobiota, zoals inflammatoire (ontsteking) darmziekten of leverziekten (hepatische encefalopathie). Daarnaast lijkt de aanwezigheid van *Blastocystis* juist geassocieerd te zijn met het hebben van een diverse en gezonde darmmicrobiota. De donoren van de NDFB worden gescreend op *Blastocystis* middels microscopie en niet met de meer gevoelige PCR (polymerase chain reaction) techniek. Doordat twee donoren en hun fecessuspensies gebruikt voor fecestransplantatie achteraf *Blastocystis* PCR positief bleken te zijn, kon een eventuele overdracht van *Blastocystis* van donor

naar patiënt onderzoeken. Door een combinatie van DNA-technieken op ontlastingsmonsters afgenomen van patiënten vóór en na de FMT en hun respectievelijke donor, beschrijven wij de eerste mens op mens transmissie van *Blastocystis* in **hoofdstuk 7**. De helft van de patiënten die *Blastocystis* positieve donorontlasting ontvangt, draagt deze drie weken nadien nog steeds. Deze transmissie had geen invloed op het succespercentage van FMT voor de behandeling van rCDI. Belangrijker bovendien is dat de overdracht niet resulteerde in gastro-intestinale symptomatologie bij de patiënten. Deze studie is een belangrijke stap op weg naar het loslaten van de uitsluiting van *Blastocystis* bevattende donorfeces.

FMT is in verschillende onderzoeken gebuikt om BRMO uit het maag-darmkanaal te verwijderen, maar de resultaten zijn nog niet heel bemoedigend. In **hoofdstuk 8** wordt het potentieel van FMT voor de eradicatie van BRMO-dragerschap onderzocht bij een patiënt die leed aan recidiverende urineweginfectie met een BRMO (carbapenemase producerende *Pseudomonas aeruginosa*). De patiënt kwam hierdoor niet in aanmerking voor een nier-pancreastransplantatie. Een FMT aansluitend op een antibiotica voorbehandeling voorkwam een recidief van de BRMO *Pseudomonas* urineweginfectie en werd de *Pseudomonas* niet meer aangetroffen in het maag-darm kanaal van de patiënt. Hoewel de behandeling een klinisch succes was, werd een gedeeltelijk microbiologisch falen waargenomen aangezien darmkolonisatie met een ESBL-producerende *E. coli* (ook een BRMO) wel persisteerde. In tegenstelling tot de zeer verstoorde darmmicrobiota bij rCDI patiënten, werd middels microbiota analyse (16S rRNA analyse) bij deze patiënt een vrijwel intacte diversiteit en samenstelling van de darmmicrobiota aangetroffen vóór transplantatie. Dit suggereert dat een intacte microbiota van de patiënt mogelijk minder vatbaar is voor vervanging van ongewenste bacteriën, en vereist eradicatie van de BRMO *E. coli* andere, complexere microbiota interventies.

Met het toenemende aantal studies die wijzen op de mogelijk gunstige effecten van FMT bij patiënten met verschillende aandoeningen binnen en buiten het maag-darm kanaal, wordt in de nabije toekomst een toenemende vraag naar zorgvuldig gescreende en goed gekarakteriseerde fecessuspensie verwacht. In het gehele proces van onderzoek, implementatie en vervolgens langdurige monitoren van de uiteindelijke uitkomsten zullen fecesbanken kunnen faciliteren. De ervaringen van dit proef-

schrift kunnen bijdragen aan het opzetten, standaardiseren en verder ontwikkelen van fecesbanken en onderzoeksinstituten van microbiota modificerende therapieën.

Toekomst microbiota modificerende therapieën

Bij de oprichting van de NDFB in 2015 werd algemeen aangenomen dat andere microbiota-modulerende therapieën (bijvoorbeeld next generation probiotics) FMT voor rCDI binnen vijf tot tien jaar zouden vervangen. Tot op heden is echter geen enkel microbiota-modulerend medicijn even effectief gebleken als FMT voor de behandeling van rCDI. Het samenstellen van een goed gekarakteriseerde bacteriële microbiota gemeenschap met het vermogen om de kolonisatie resistentie tegen *C. difficile* infectie te herstellen bij een patiënt, is veel moeilijker dan eerder werd aangenomen. Het lijkt erop dat de kracht van FMT bestaat uit de transplantatie van een compleet en werkend ecosysteem. Tegelijkertijd schuilt hierin ook het gevaar, namelijk het transplanteren van een compleet, maar ongecontroleerd, ongestandaardiseerd en niet volledig begrepen ecosysteem. Dit brengt theoretisch het risico mee op overdracht van een andere microbiota geassocieerde aandoening. Gelukkig blijken deze risico's tot nu toe verwaarloosbaar, al kunnen de langetermijngevolgen nog niet compleet worden overzien. Op dit moment blijft FMT als ruwe diamant de gouden standaard. Voor de ontwikkeling van meer geavanceerde precisie-microbiota therapieën, zal FMT de weg plaveien door mechanistische inzichten te verschaffen in de effecten van de getransplanteerde donor microbiota op de specifieke ziekte. In de toekomst staat bij voorkeur een arsenaal van verschillende microbiota-precisie therapieën tot onze beschikking voor diverse ziekten, welke afhankelijk van de specifieke verstoring van de microbiota op maat kunnen worden toegediend als gepersonaliseerde behandeling.

List of publications

Microbiota-associated risk factors for asymptomatic gut colonization with multidrug resistant organisms in a Dutch nursing home

Q.R. Ducarmon*, **E.M. Terveer***, S. Nooij, M.N. Bloem, K.E.W. Vendrik, M.A.A. Caljouw, I.M.J.G. Sanders, S.M. van Dorp, R.D. Zwittink*, E.J. Kuijper*
* Both authors contributed equally to this work

Genome Medicine. 2021. 13(1):54 doi: 10.1186/s13073-021-00869-z.PMID: 33827686

Ten year follow-up of patients treated with fecal microbiota transplantation for recurrent Clostridioides difficile infection from a randomized controlled trial and review of the literature

R.E. Ooijevaar, E. van Nood, A. Goorhuis, **E.M. Terveer**, J. van Prehn, H.W. Verspaget, Y.H. van Beurden, M.G.W. Dijkgraaf, J.J. Keller

Microorganisms. 2021. 6;9(3)548. doi: 10.3390/microorganisms9030548.

Periodic screening of donor faeces with a quarantine period to prevent transmission of multidrug resistant organisms during faecal microbiota transplantation: a retrospective cohort analysis

K.E.W. Vendrik, **E.M. Terveer**, E.J. Kuijper, S. Nooij, E. Boeije-Koppenol, I.M.J. Sanders, E. van Lingen, H.W. Verspaget, E.K.L. Berssenbrugge, J.J. Keller*, J. van Prehn*
*Both authors contributed equally to this work

Lancet Infectious diseases. 2020. in press. doi: 10.1016/s1473-3099(20)30473-4

A standardised model for stool banking for faecal microbiota transplantation: a consensus report from a multidisciplinary UEG working group

J.J. Keller, R.E. Ooijevaar, C.L. Hvas, **E.M. Terveer**, S.C. Lieberknecht, C. Högenauer, P. Arkkila, H. Sokol, O. Gridnyev, F. Mégraud, P.K. Kump, R. Nakov, S.D. Goldenberg, R. Satokari, S. Tkatch, M. Sanguinetti, G. Cammarota, A. Dorofeev, O. Gubska, G. Ianiro, E. Mattila, R.P. Arasaradnam, S.K. Sarin, A. Sood, L. Putignani, L. Alric, S.M. Baunwall, J. Kupcinskas, A. Link, A.G. Goorhuis, H.W. Verspaget, C. Ponsioen, G.L. Hold, H. Tilg, Z. Kassam, E.J. Kuijper, A. Gasbarrini, C.J. Mulder, H.R. Williams, M.J. Vehreschild

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Faecal microbiota transplantation for Clostridioides difficile infection: Four years' experience of the Netherlands Donor Feces Bank

E.M. Terveer, K.E. Vendrik, R.E. Ooijevaar, E. van Lingen, E. Boeije-Koppenol, E. van Nood, A. Goorhuis, M.P. Bauer, Y.H. van Beurden, M.G. Dijkgraaf, C.J. Mulder, C.M. Vandenbroucke-Grauls, J.F.M.L. Seegers, J. van Prehn, H.W. Verspaget, E.J. Kuijper, J.J. Keller. 2020

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The Bacterial Gut Microbiota of Adult Patients Infected, Colonized or Noncolonized by *Clostridioides difficile*

M.J.T. Crobach, Q.R. Ducarmon, **E.M. Terveer**, C. Harmanus, I.M.G.J. Sanders, K.M. Verduin, E.J. Kuijper, R.D. Zwittink.

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*Both authors contributed equally to this work

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Curriculum vitae

Liz Terveer was born on September 15th, 1984 in Klaaswaal, the Netherlands. After graduating at Luzac college in Rotterdam, she attended the University of Leiden in 2002 to study Biology. In 2005 she received a bachelor degree in Biology. The molecular and human medicine part of the Biology study was very challenging and exciting, so she switched to a Biomedical Sciences master which was subsequently followed with a Medicine study. In 2011, Liz received both her Biomedical sciences Masters and Medical degree of the University of Leiden. During her Biomedical sciences internship the passion for microbiology and *Clostridioides difficile* was sparked at the Experimental Bacteriology of the Department of Medical Microbiology at the Leiden University Medical Center. At the same department her training as medical microbiologist started in 2011. Four years later in 2015, Liz was involved in the establishment and development both of the Netherlands Donor Feces Bank (NDFB) and subsequently the Center for Microbiota Analysis and Therapeutics (CMAT) in 2017, which became the starting points of this thesis. Under supervision of prof. dr. Ed Kuijper and dr. Josbert Keller, Liz started to coordinate the Netherlands Donor Feces Bank (NDFB) and to investigate the role of the microbiota in the defense against multidrug resistant Gram negative bacteria and *C. difficile*. She completed her residency in 2016 and since then works as a registered medical specialist with a focus on Bacteriology. Together with Bas Huismans, Liz has two children; Olivier and Seppe. Liz became chairman of the executive board of the NDFB in 2020 and supervises all activities, varying from donor screening and patient treatment with feces microbiota transplantations (FMT), to analyses of the microbiota and studies for new applications of FMT for various diseases.

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